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APPELLANTS' BRIEF ON APPEAL PURSUANT TO 37 C.F.R. § 41.37

In support of Appellants' Notice of Appeal that was filed in connection with the above-captioned case on November 21, 2008, and with reference to the Office Action that was mailed in this case on August 20, 2008, submitted herewith is Appellants' Appeal Brief.

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Real Party in Interest

The real party in interest in this case is The McLean Hospital Corporation, to whom all interest in the present application has been assigned.

Related Appeals and Interferences

There are no appeals or interferences related to this case.

Status of Claims

Claims 1-3, 5, 7-20, and 22-26 are pending and on appeal. Claims 4, 6, 21, and 27-30 have been cancelled. Claims 1-3, 5, 7-20, and 22-26 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. Claims 1-3, 5, 7-13, 17, 19, 22, 23, and 26 stand rejected under 35 U.S.C. § 112, second paragraph. Claims 17 and 22 are objected to for informalities. Claims 1-3, 5, 7-20, and 22-26 stand further rejected for obviousness-type double patenting over claims 1-16 of U.S. Patent No. 6,103,703 (hereafter “Renshaw”) and under 35 U.S.C. § 103 for obviousness over Renshaw.

Status of Amendments

An amendment to claims 1, 12, 17, and 22 under 37 C.F.R. § 41.33(a) was filed on June 16, 2009 to address one basis for rejection of claims 1, 12, and 22 under 35 U.S.C. § 112, second paragraph and to address an objection to claims 17 and 22. Entry of this amendment is being considered.

Summary of Claimed Subject Matter

The invention features a method of normalizing the sleep/wake cycle of a mammal not suffering from insomnia by administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine

diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine (Specification, page 1, lines 27-30; page 2, lines 6-8; page 7, lines 24-27; and page 9, lines 16-18).

The invention further features a method of treating a sleep disorder in a mammal not suffering from an existing physical condition or insomnia by administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine (Specification, page 2, lines 3-8; page 5, lines 16-19; page 7, lines 24-27; and page 9, lines 16-18).

The invention also features a method of increasing cognitive function in a sleep-deprived mammal not suffering from insomnia by administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Specification, page 2, lines 6-8; page 2, lines 11-14; page 7, lines 24-27; page 9, lines 1-7; and page 9, lines 16-18).

Finally, the invention features a method of treating a sleep disorder other than insomnia or sleep apnea by administering to a mammal a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP),

deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, dipyridamole, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Specification, page 2, lines 3-8; page 7, lines 24-27; and page 9, lines 16-18).

#### Grounds of Rejection to be Reviewed on Appeal

Claims 1-3, 5, 7-20, and 22-26 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. Claims 1-3, 5, 7-13, 17, 19, 22, 23, and 26 stand rejected under 35 U.S.C. § 112, second paragraph.<sup>1</sup> Claims 1-3, 5, 7-20, and 22-26 stand further rejected for obviousness-type double patenting over claims 1-16 of Renshaw and under 35 U.S.C. § 103 for obviousness over Renshaw.

#### Argument

##### *Enablement*

The first issue presented on appeal involves the rejection of claims 1-3, 5, 7-20, and 22-26 for lack of enablement. As stated in M.P.E.P. § 2164, “[t]he purpose of the requirement that the specification describe how to make and use the claimed invention is to ensure that the invention is communicated to the interested public in a meaningful way.” In considering the adequacy of a disclosure, M.P.E.P. § 2164.04 requires:

A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as being in compliance with the enablement requirement ..., unless there is a reason to doubt the objective truth of the statements contained therein... (emphasis added)

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<sup>1</sup> Claims 1-2, 13, 17, 19, 22, and 23 are noted as rejected on page 7 of the Action, and claims 3, 5, 7-16, 18, 20, 21 and 24-26 are additionally noted as rejected on page 10 of the Action.

Furthermore, the Office is required to “back up assertions of its own with acceptable evidence or reasoning which is *inconsistent* with the contested statement.” (M.P.E.P. § 2164.04; emphasis added). Such evidence or reasoning has not been provided by the Examiner to support the rejection of the present claims.

### **I. The Present Claims Are Enabled by the Specification.**

The application includes four independent claims, claims 1, 12, 17, and 22. These claims read as follows:<sup>2</sup>

1. A method of normalizing the sleep/wake cycle of a mammal in need thereof, said method comprising orally administering to said mammal a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal is not suffering from insomnia.

Claim 12 reads:

12. A method of treating a sleep disorder, said method comprising administering to a mammal in need thereof a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal’s health is not compromised because of an existing physical condition and wherein said mammal is not suffering from insomnia.

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<sup>2</sup> The claims are those as amended on June 16, 2009. The claims prior to amendment are provided in the Claims Appendix.

Claim 17 reads:

17. A method of increasing cognitive function in a sleep-deprived mammal, said method comprising administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) to a mammal suffering from sleep deprivation, wherein said mammal is not suffering from insomnia.

Claim 22 reads:

22. A method of treating a sleep disorder, said method comprising administering to a mammal in need thereof a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, dipyridamole, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), wherein said sleep disorder is not insomnia or sleep apnea.

Each of these claims is directed to treatment of a disorder or condition associated with sleep in specified classes of mammals by employing one of the listed classes of compounds.

The present invention is based on the discovery by the inventor that citicoline, i.e., CDP-choline, is useful for the normalization of the sleep/wake cycle and improves quality of sleep and mood. The specification describes experiments performed by the inventors on human subjects at page 7, lines 1-4 and Figure 1 and page 7, lines 15-21 and Figures 2A and 2B. As is further noted in the specification, based on these experimental

observations, citicoline likely stabilizes homeostatic processes involved in numerous sleep disorders (Specification, page 6, lines 23-26) and may be used to increase cognitive functioning in subjects in a sleep-deprived state (Specification, page 6, line 26 – page 7, line 1).

The specification further teaches that citicoline is metabolized into cytidine and choline and that cytidine interconverts with uridine in vivo, thereby supporting the claimed uses of these additional classes of compounds (Specification, page 8, lines 3-7). The specification also teaches that adenosine-containing or elevating compounds may similarly be capable of maintaining sleep homeostasis and are accordingly useful in the claimed methods (Specification, page 8, lines 19-27). Finally, the specification teaches that creatine may be useful in the methods of the invention, as it increases levels of ATP (Specification, page 9, lines 22-25).

Moreover, the specification provides examples of specific compounds that may be employed in the claimed methods (Specification, pages 7-9) and describes exemplary doses, formulations, and routes of administration for these compounds (Specification, pages 9-11).

Accordingly, the specification provides experimental data and additional reasoning that would lead one skilled in the art to conclude that a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) is effective in the claimed methods. Further, the specification provides a teaching with respect to administration of these compounds for these methods.

Thus, one skilled in the art, i.e., a medicinal chemist or physician, could practice the present invention without undue experimentation. And the scope of enablement is fully supported by sound scientific experiments – indeed, experimental data of a human patient – and sound scientific reasoning.

## **II. The Rejection Is Unsupported by Relevant Facts or Applicable Law.**

Contrary to Appellants' evidence and sound scientific reasoning in support of the enablement of the claimed methods, the Examiner has failed to provide any appropriate legal or factual basis for the present rejection. The Examiner's position on the lack of enablement of the present claims does not rely on a scientific reference or scientific fact for support but instead relies on personal opinion (Office Action mailed on August 20, 2008, "Action," pages 2-6). As is discussed in detail below, *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988) is the controlling law on enablement, and this decision and the M.P.E.P. sections summarizing it (M.P.E.P. § 2164) confirm that any rejection based on scope of enablement requires the Examiner to make findings of fact and reach a conclusion on enablement only after weighing these facts (see, M.P.E.P. § 2164.01(a), citing *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404). Furthermore, as discussed above, the initial burden is on the Office to provide, at a minimum, specific technical reasons to question the enablement provided in the specification (M.P.E.P. § 2164.04 and citations therein). In the present case, the basis for the rejection is the Examiner's unsupported belief that Appellants have not provided sufficient data to support the claimed scope. The Examiner's position is therefore procedurally insufficient.

In addition to the failure to provide factual basis for the rejection, the Examiner misapprehends the burden on the Office in raising such a rejection. During prosecution, Appellants repeatedly pointed out that the Examiner failed to provide any factual or legal support for the rejection and that he therefore failed to establish a *prima facie* case of unpatentability (see, e.g., Replies filed November 3, 2006 and February 7, 2008). In response to such arguments, the Examiner states: "[A]pplicant again argues in a manner

suggesting [that] the burden is on the Office to adequately support the instant rejection.” (Action, page 6). As discussed above and in contrast to the Examiner’s position, the Office *always* bears the initial burden of establishing a *prima facie* case of unpatentability, no matter the type of rejection raised (see, also, M.P.E.P. § 2107.02 (IV), “[T]he examiner bears the initial burden, on review of the prior art or on any other ground, of presenting a *prima facie* case of unpatentability.” *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Accordingly, it is reversible error for the Examiner to refuse to support the rejection.

The Examiner further relies on a Board decision, i.e., *Ex parte Balzarini*, 21 USPQ2d 1892 (BPAI 1991), and inapplicable sections of the M.P.E.P. to support the position that “claims directed to medicinal treatments of diseases in highly unpredictable art areas are properly rejected under 35 U.S.C. § 112, first paragraph as lacking adequate enablement, in the absence of sufficient test data in support of the efficacy of the alleged treatment. See MPEP at §2107.03.” (Action, page 6; emphasis in original). As has been previously argued by Appellants (see, Replies filed November 3, 2006 and July 23, 2007), both the portion of *Ex parte Balzarini* relied upon by the Examiner and M.P.E.P. § 2107.03 involve rejections for lack of utility, not scope of enablement. Furthermore, *Ex parte Balzarini* is a *nonprecedential* opinion of the Board and does not have the force of rule or law. Although the Examiner has stated on the record that the present rejection is not a utility rejection (Interview Summary, June 20, 2006), the scope of enablement rejection continues to be based on decisions and guidance specifically for utility rejections (see, Action, page 6 and Action mailed January 19, 2007, pages 4 and 6). This decision and section of the M.P.E.P. are simply not relevant to a consideration of the scope of enablement of the present claims, and the Examiner’s reliance on them is misplaced. Furthermore, *Ex parte Balzarini* and M.P.E.P. § 2107.03 do not stand for a general proposition that a patent application must provide test data for a method of medical treatment or that Appellants must provide test data at the request of an Examiner. Instead, they confirm Appellants’ position that the initial burden is on the Office to

establish a *prima facie* case of unpatentability (see, e.g., *Balzarini*, 21 USPQ2d at 1895.). In addition, when data are provided in an application, as in the present case, an Examiner must “evaluate all the facts and evidence and state why one would not expect to be able to extrapolate that one example across the entire scope of the claims.” (M.P.E.P. § 2164.02) In the present case, the Examiner has reversed the standard, stating:

[E]xaminer notes that in medicinally directed claims, the burden is on applicant to provide adequate evidentiary support for the claimed method of medicinal treatment.

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Applicant had been and is respectfully requested to supply additional data to demonstrate that the allegations above are properly extrapolated from the single data point provided. (Action, page 6).

Finally, we note that, while a rejection for lack of utility was upheld by the Board in *Ex parte Balazarini*, that case involved a rejection supported by scientific evidence in the form of published references indicating that the claimed methods were inoperable (see, e.g., *Balazarini*, 21 USPQ2d at 1896). No such evidence has been provided in the present application.

In view of the Examiner’s refusal to provide any scientific support for the rejection and his reliance on inapplicable legal standards, the rejection should be reversed.

### **III. Proper Consideration of the *Wands* Factors Shows that the Claims Are Enabled.**

The controlling case in this area, *In re Wands* (858 F.2d at 737, 8 USPQ2d at 1404) provides factors to be considered in determining whether claims are enabled. In maintaining the rejection, the Examiner discusses each of the *Wands* factors and concludes “the minimum necessary guidance concerning how to use the various different active ingredients …, and their application to various different sleep disorder treatments, is simply absent.” (Action, page 4). As is discussed below, however, the Examiner’s position is based on opinion and is not supported by references or technical reasoning.

Furthermore, the Examiner typically groups all of the claims without regard to the distinct methods recited therein.

During prosecution, the claims were twice rejected for lack of enablement. The first rejection is found on pages 2-3 of the August 20, 2008 Action, and the second is found on pages 3-6 of the August 20, 2008 Action. In the first rejection, the Examiner focuses solely on the human data provided in the present specification and suggests additional experiments. In the second rejection, the Examiner considers the *Wands* factors. Because *In re Wands* is the controlling law on enablement, Appellants have addressed the first rejection as it relates to the *Wands* analysis. Appellants address each *Wands* factor in turn as follows.

*(A) Breadth of the Claims.*

In considering this factor, the Examiner states:

[T]he breadth of many of the claims is excessive because of the presence of generic terms including “treating a sleep disorder” (claims 1, 12 and 22) and “increasing cognitive function” (claim 17). (Action, page 3, formatting omitted)

This statement merely concludes that the scope is excessive because the claims recite generic terms such as “treating a sleep disorder” and “increasing cognitive function.” As noted above, a *Wands* analysis requires findings of fact. Here, the Examiner has made no factual finding other than noting that the claims are generic. Appellant is unaware of any legal standard for the proposition that use of a generic term in a claim results in the breadth being *per se* excessive, and the Examiner has provided no legal precedent to support his position. Furthermore, claim 1 is directed to a method of normalizing the sleep/wake cycle, which the Examiner fails to address.

Moreover, while the claims are generic, Appellants have provided exemplary embodiments of the individual conditions, numerous compounds to achieve the claimed effects, exemplary doses, formulations, and routes of administration of these compounds, exemplary patient populations, sound scientific argument to support the effectiveness of

the claimed compounds for the full scope as claimed, and methods of assessing therapeutic efficacy, as discussed above. Therefore, Appellants have provided a disclosure that bears a reasonable correlation to the scope of the claims (see, e.g., M.P.E.P. § 2164.08 and citations therein).

*(B) Nature of the Invention.*

The Examiner correctly notes that the claims are directed to treatment of sleep or sleep-related disorders (Action, page 3). For reasons not apparent from the record, the Examiner appears to limit the nature of the treated sleep-related disorders to those listed in claims 13, 15, 20, and 25 (Action, page 3). Appellants emphasize that claims are directed to normalization of the sleep/wake cycle, treatment of particular sleep disorders, and increasing cognitive function in a sleep-deprived mammal.

*(C) The State of the Prior Art.*

In characterizing the prior art, the Office states only that “CDP-choline is associated in some prior art references with the effective amelioration of insomnia, particularly in elderly hosts.” (Action, page 3). As with the breadth of the claims, the Examiner fails to comment on all of the claimed methods. Appellants note that compounds recited or encompassed by the instant claims are reported to have pharmaceutical activity in Renshaw (discussed below) and other references previously cited against the claims during prosecution, e.g., Yamamoto et al. (U.S. Patent No. 5,635,486); Wurtman et al. (U.S. Patent Application Publication No. US 2003/0114415); Fernandez (*Arzneimittelforschung. Drug Res.* 33:1073-1080 (1983)); Ferrer Internacional, S.A. (International Publication No. WO 01/72288); Radulovacki et al. (*J. Pharmacol. Exper. Ther.* 228:268-274 (1984)); and Satoh et al. (*Euro. J. Pharmacol.* 351:155-162 (1998)) (Evidence Appendix). These references also provide background on treatment of other sleep-related disorders. While Appellants have distinguished each of these cited references from the instant claims, these references provide general guidance

to one skilled in the art, when combined with the discovery of the instant disclosure. Appellants also emphasize that the Examiner has not provided a single reference calling into question the feasibility of normalizing the sleep/wake cycle, treating a sleep disorder, or increasing cognitive function in a sleep-deprived mammal, or the feasibility of using any of the compounds recited by the present claims.

*(D) The Level of One of Ordinary Skill.*

On this point, the Examiner states:

the level of the ordinary practitioner is variable, because the administration of CDP-choline has been shown herein to be effective in one host, but the remainder of the claimed active ingredients have not been shown herein to have similar activities" (Action, page 3).

The Examiner has erred in considering this factor, as his analysis is based on his interpretation of the level in view of the present specification rather than the art as a whole (see, e.g., M.P.E.P. § 2164.05).

Appellants assert that the level of one of ordinary skill in this art is equivalent to a Ph.D. level medicinal chemist or a medical doctor. Accordingly, these individuals have a high level of skill in the pharmaceutical arts. As discussed above, Appellants have provided exemplary embodiments of the individual conditions, numerous compounds to achieve the claimed effects, exemplary doses, formulations, and routes of administration of these compounds, exemplary patient populations, sound scientific argument to support the effectiveness of the claimed compounds for the full scope as claimed, and methods of assessing therapeutic efficacy. Accordingly, one of ordinary skill could practice the full scope of the present claims because optimization of these parameters, once provided by Appellants, is routine in the art.

*(E) The Level of Predictability in the Art.*

With respect to this prong, the Examiner merely states that the art of treating sleep disorders is highly variable in its predictability because of the large array of different causes or circumstances under which it is observed to occur (Action, pages 3-4). Again, the Examiner has grouped all claims together rather than taking into account the distinctions of each independent claim. Further, no evidence of the asserted unpredictability has been provided. As discussed above, Appellants' specification includes experimental data and scientific reasoning to support the effectiveness of the claimed compounds for the full scope as claimed. Furthermore, Yamamoto et al., Wurtman et al., Fernandez, Ferrer Internacional, S.A., Radulovacki et al., and Satoh et al. (Evidence Appendix) were cited by the Examiner and discuss conditions related to those instantly claimed. These references indicate that the claimed methods are directed to identifiable subject matter and that treatments are possible, thereby contradicting the Examiner's assertion of general unpredictability.

*(F) The Amount of Direction Provided by the Inventor.*

For this prong, the Examiner has confused "direction" with experimental data and only considered Figures 1 and 2 of the present application (Action, page 4). In addition to the human data shown in these Figures, Appellants provide lists of specific compounds for use in the methods of the invention, specific disorders and conditions for treatment, preferred dosages, formulations, and routes of administration of these compounds, exemplary patient populations, sound scientific argument to support the effectiveness of the claimed compounds for the full scope as claimed, and methods of assessing therapeutic efficacy. Given this disclosure and the prior art, nothing more is required to allow the skilled artisan to practice the invention.

*(G) The Existence of Working Examples.*

In commenting on this factor, the Examiner acknowledges that Appellants have provided a working example, but dismisses the data as applicable to only a single subject and as not specifying the particular disorder being treated (Action, page 4). The Examiner has also expressed concern that the data provided in the specification are not applicable to people in general because the subject of certain experiments was a drug user (Action, page 2). Appellants disagree with the Examiner's conclusions.

As is stated in the specification on page 7, Figure 1 shows data indicating that CDP-choline improves sleep quality, and Figures 2A-2B show the normalization of the sleep/wake cycle of a cocaine user after administration of CDP-choline. The data in Figure 1 are on the quality of sleep for multiple subjects (who were also monitored as a result of cocaine use). Thus, the specification provides supporting data on the ability of CDP-choline to affect sleep for more than one individual, in contrast to the assertion of the Examiner. Figure 2A shows the activity levels of a subject over five days prior to administration of CDP-choline, and Figure 2B shows the activity levels of the same subject over five days after treatment with CDP-choline (Specification, page 7, lines 15-21). The specification further states that CDP-choline was effective in normalizing the sleep/wake cycle of the subject of Figures 2A-2B (Specification, page 7, lines 17-19). Thus, the condition being treated, i.e., an abnormal sleep/wake cycle, is apparent, in contrast to the assertion of the Examiner. Although the subjects of the experiments represented in Figures 1 and 2A-2B were users of cocaine, Appellants also submit that the results of CDP-choline on the sleep/wake cycle and quality of sleep are applicable to individuals who are not users of cocaine.

As Appellants have provided working examples and explanations of their relevance, the Examiner must "evaluate all the facts and evidence and state why one would not expect to be able to extrapolate that ... example across the entire scope of the

claims" (M.P.E.P. § 2164.02). Throughout prosecution of this application, the Examiner has failed to do so. Instead, the Examiner merely concludes:

In any event the instant data set is simply inadequate to enable the instant patent claims because of the lack of adequate showing(s) that the claimed effects of CDP-choline administration are common to a reasonable number of similarly situated hosts in need of such treatment. (Action, page 2)

The Examiner has not, however, supported this conclusion with legal or scientific analysis as required. Furthermore, as discussed above, the Examiner has failed to explain why the human clinical data provided in the specification are insufficient to support the enablement of the full claim scope. Finally, the Examiner has provided no reason why the data on the observed effect of CDP-choline on the sleep-wake cycle and sleep quality in cocaine users are not applicable to other subjects.

There is no better indicator of the enablement of the present claims than the demonstrated efficacy in humans, as provided in the present specification. These data have, however, been dismissed by the Examiner, who requests that Appellants conduct further clinical trials (see, Action, pages 2 and 6). The enablement standard does not require a clinical trial suitable for submission to the FDA (see M.P.E.P. §§ 2107.03(IV)-(V), to the extent the rejection is based on lack of utility, and 2164.05); indeed, it does not require experimental data of any kind (M.P.E.P. § 2164.02). Accordingly, the Examiner's continued insistence that Appellants conduct clinical trials is inappropriate.

Moreover, as discussed above, Appellants provide scientific reasoning in the specification to support the effectiveness of the claimed compounds for the full scope as claimed. In particular, the specification teaches that, based on the experimental observations, CDP-choline likely stabilizes homeostatic process involved in numerous sleep disorders (Specification, page 6, lines 23-26) and may be used to increase cognitive functioning in subjects in a sleep-deprived state (Specification, page 6, line 26 – page 7, line 1). The specification further provides scientific reasoning supporting the use of uridine-containing compounds, adenosine-containing or elevating compounds, and

creatine in the claimed methods (Specification, page 8, lines 19-27; page 9, lines 14-16; and page 9, lines 22-25).

*(H) The Quantity of Experimentation Needed to Make or Use the Invention Based on the Content of the Disclosure.*

For this factor, the Examiner concludes that the quantity of experimentation is excessive “in light of the indefiniteness and functional claim terminology” and “because the exemplary evidence is so limited in quantity” (Action, page 4). Again, the Examiner has failed to make any findings of fact to support this conclusion. The Examiner has also only considered the experimental data provided and not the scientific reasoning or exemplary conditions, compounds, dosages, formulations, routes of administration, patient populations, and assay methods provided. As is discussed below, the instant claims, though generic, are definite because one skilled in the art would understand their metes and bounds.

In the present case, Appellants have provided data on the efficacy of CDP-choline in human subjects as well as scientific reasoning to support the scope of the claims. In addition, the specification provides exemplary assays for determining the effectiveness of various compounds, a defined list of specific compounds, preferred dosages, formulations, and patient populations. In the pharmaceutical arts, experimentation to determine the optimum chemical composition, formulation, and dosage for treating a particular condition is routine once the lead compounds are identified. Accordingly, Appellants have provided a reasonable amount of guidance with respect to the claimed methods.

In short, a consideration of the *Wands* factors indicates that the instant claims are enabled. The claims are supported by human data and substantial guidance in the specification, and, as evidenced by the prior art, the claims are directed to known,

tractable problems. In view of these facts and the lack of any contradictory reasoning or evidence from the Examiner, the rejection should be reversed.

#### **IV. Additional Factors Support the Enablement of Several of the Dependent Claims.**

In addition to the arguments above for the independent claims, additional considerations support the enablement of claims 3, 5, 7, 9, 13, 16, 18, 23, and 26.

In particular, claims 3, 5, and 7 recite specific compounds, i.e., cytidine, CDP-choline, and CDP, for use in the method of claim 1; claim 16 recites CDP-choline for use in the method of claim 12; claim 18 recites CDP-choline for use in the method of claim 17; and claim 26 recites CDP-choline for use in the method of claim 22. As each of these claims is directed to the use of specific compounds, their scope is narrower than that of the independent claims. In addition, claims 5, 16, 18, and 26 require CDP-choline, which is the active agent used to obtain the experimental data provided in the specification. As discussed above, the specification teaches that, based on these experimental observations, CDP-choline (citicoline) likely stabilizes homeostatic processes involved in numerous sleep disorders (Specification, page 6, lines 23-26) and may be used to increase cognitive functioning in subjects in a sleep-deprived state (Specification, page 6, line 26 – page 7, line 1). The specification further teaches that citicoline is metabolized into cytidine and choline and that cytidine interconverts with uridine in vivo, thereby supporting the claimed uses of these additional classes of compounds (Specification, page 8, lines 3-7 and page 9, lines 14-16).

Claims 13 and 23 are directed to treatment of sleep disorders caused by substance abuse disorders. The human subjects of the experiments provided in the specification were users of cocaine (Specification, page 7, lines 15-17, Figures 1 and 2A-2B). The administration of CDP-choline to a representative individual resulted in the normalization of the sleep/wake cycle (Specification, page 7, lines 17-19). As this individual had a disrupted sleep/wake cycle as a result of cocaine abuse, these data provide direct

evidence of the efficacy of the claimed methods of treating sleep disorders caused by a substance abuse disorder.

Finally, claim 9 is directed to the normalization of the sleep/wake cycle in a human subject. As discussed, the specification provides data showing the efficacy of the claimed compounds in normalizing the sleep/wake cycle of a human subject. Accordingly, the data directly support the enablement of this claim.

Claims 3, 5, 7, 9, 13, 16, 18, 23, and 26 are directed to particular embodiments of the independent claims. Each of these claims is directed to a compound, cause of the disorder, or subject evaluated experimentally. In addition to the general failure to support the rejection discussed above, the Examiner has failed to provide any reason why the data do not support the enablement of these claims. For these reasons as well, the rejection should be reversed.

#### *Definiteness*

The Examiner has also rejected claims 1-3, 5, 7-13, 17, 19, 22, 23, and 26 for indefiniteness. The purpose of the definiteness requirement is to ensure that “the scope of the claim is clear to a hypothetical person possessing the ordinary skill in the pertinent art” (M.P.E.P. § 2171). An applicant “can define in the claims what they regard as their invention essentially in whatever terms they choose... [and] may use functional language, alternative expressions, negative limitations, or any style of expression or format of claim which makes clear the boundaries of the subject matter for which protection is sought” (M.P.E.P. § 2173.01). In addition, “a claim may not be rejected solely because of the type of language used to define the subject matter for which patent protection is sought” (M.P.E.P. § 2173.01).

On the issue of definiteness, M.P.E.P. § 2173.02 states:

Definiteness of claim language must be analyzed, not in a vacuum, but in light of:

- (A) The content of the particular application disclosure;
- (B) The teachings of the prior art; and

(C) The claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made.

Many of the rejections raised by the Examiner turn on his belief that certain claim terms are too broad. On this issue, the law is clear that “[b]readth of a claim is not to be equated with indefiniteness” (M.P.E.P. § 2173.04).

Each of the bases for rejection is addressed in turn below.

#### **I. Claims 1, 12, 17, and 22 – “compound comprising”**

The first basis for this rejection is that claims 1, 12, 17, and 22 recite:

compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine<sup>3</sup>

On this point, the Examiner states:

the subsequent list of compounds are all named as separate compounds rather than substituent moieties of a larger molecular species, and because the larger molecular species implied by the term “comprising” (including) is not subsequently defined thereby leaving the metes and bounds of the claimed subject matter incompletely defined. (Action, page 7)

Notably, the Examiner has pointed to no precedent or rule that prohibits use of the language recited in the present claims and has provided no reason or evidence as to why one skilled in the art would be unable to discern the metes and bounds of the claims.

The present claims recite several classes of compounds, the members of which are related by their formal relationship to the recited compound. On this point, M.P.E.P. § 2173.05(t) states, “Chemical compounds may be claimed by a name that adequately

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3. Claims 17 and 22 additionally recite creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA).

describes the material to one skilled in the art,” and, here, one skilled in the art would understand the metes and bounds of the claims based on the formal relationship between the compounds encompassed and the recited classes. In addition, the definiteness requirement does not require that each chemical entity encompassed by a claim be described in complete molecular detail, but instead only in such detail as required for one skilled in the art to determine the scope of the claims. Classes of compounds, as with the present claims, are routinely used to refer to numerous related compounds both in the scientific and the patent literature. For example, von Borstel (International Publication No. WO 00/11952) (Evidence Appendix) provides acylated forms of cytidine and uridine on pages 9 and 10, and Radulovacki et al. describes several adenosine analogs including cyclohexyladenosine and adenosine-5'-N-ethylcarboxamide. One skilled in the art viewing these compounds would readily determine that they comprise cytidine, uridine, or adenosine, as recited in the present claims. The rejection should be reversed.

## **II. Claim 12 – “physical condition”**

Claim 12 stands further rejected for indefiniteness for reciting that the “mammal’s health is not compromised by an existing physical condition.” The basis of this rejection is that the limitation “is an improper negative limitation because the particular ‘existing physical limitation[s]’ have not been specified in the claim.” (Action, page 7). The Examiner also asserts that “one of ordinary skill cannot determine from the claim what existing medical conditions are included within the metes and bounds of the claims and what existing medical conditions are not so included by the instant claims...” (Action, page 8).

First, the Examiner has based his analysis on terms not recited in the claims. Specifically, claim 12 does not use the term “medical conditions,” as asserted by the Examiner. Instead, the claim excludes treatment of mammals whose health is compromised by a *physical* condition, e.g., head trauma. The claim does not exclude

treatment of mammals suffering from *mental* conditions, such as the sleep disorders being treated, depression, or substance abuse.

In addition, as is noted above, breadth is not indefiniteness. Accordingly, it is improper to reject a claim for indefiniteness because it recites a generic term rather than the species that make up the genus. As argued during prosecution, the term “physical condition” is an art accepted generic term, as evidenced by a PubMed search for the term yielding 1195 references published before the present priority date (Evidence Appendix). No further specificity is thus required under § 112, second paragraph.

Negative limitations, as recited in claim 12, also do not require specifying every species of an excluded generic term. M.P.E.P. § 2173.05(i) states. “The current view of the courts is that there is nothing inherently ambiguous or uncertain about a negative limitation. So long as the boundaries of the patent protection sought are set forth definitely, albeit negatively, the claim complies with [the definiteness requirement].” As the term “physical condition” is definite, its exclusion from claim 12 is also definite.

Finally, a medical professional would know whether or not a particular subject has compromised health because of a physical condition. Claim 12 excludes treatment of sleep disorders in a mammal whose health is compromised by *any* physical condition. There is no need to specify particular disorders as they are all excluded. As one skilled in the art would understand the metes and bounds of the term “physical condition,” the rejection should be reversed.

### **III. Claims 13 and 23 – antecedent basis**

Claims 13 and 23 stand rejected for lack of antecedent basis for the phrase “said sleep disorder is caused by a substance abuse disorder.” The basis of the rejection is that the Examiner believes that specifying the cause of the sleep disorder being treated in claim 13 expands the subject matter of claim 12 (Action, page 8). Appellants disagree.

As has been argued numerous times, claim 13 is drafted according to well-established U.S. patent practices. Claim 13 is not broader than claim 12, as asserted by

the Examiner, because claim 13 places limits on terms recited in claim 12. Claim 12 covers the treatment of all sleep disorders except insomnia and those caused by physical conditions. Claim 13 covers treatment of sleep disorders caused by a substance abuse disorder. Stated another way, if the sleep disorder is not caused by a substance abuse disorder, its treatment is not covered by claim 13 but is covered by claim 12. The same reasoning applies to the dependency of claim 23 from claim 22.

No amendment of claim 13 or claim 23 is necessary to provide proper antecedent basis or to make it properly dependent from claim 12 or claim 22. The rejection should be reversed.

#### **IV. Claims 19 and 13 – “substance abuse disorder”**

Claim 19 stands rejected for reciting “not caused by a substance abuse disorder.” The Examiner bases this rejection on his belief that the limitation “renders the claim incomplete because the particular substance abuse disorder(s) has(have) not been specified.” (Action, page 8).

As discussed above with respect to claim 12, negative limitations do not require specifying every species of an excluded generic term (M.P.E.P. § 2173.05(i)). As previously argued, the term “substance abuse disorder” is an art recognized term, as evidenced by a PubMed search for the term yielding 60 references published before the present priority date (Evidence Appendix). As the term “substance abuse disorder” is definite, its exclusion from claim 19 is also definite.

As with claim 12, a medical professional would know whether or not a particular sleep disorder is caused by a substance abuse disorder. Claim 19 excludes treatment of a sleep disorder caused by *any* substance abuse disorder. There is no need to specify particular disorders as they are all excluded. As one skilled in the art would understand the metes and bounds of the term “substance abuse disorder,” the rejection should be reversed.

The Examiner also states that claim 13 is incomplete because “the particular ‘substance abuse disorder’ has not been specified.” (Action, page 8). In reply to previous arguments against the necessity of listing every substance abuse disorder in claim 13, the Examiner states that this argument “is beside the point of the above rejection which was noting the lack of antecedent basis.” (Action, page 8). To the extent that the breadth of the term “substance abuse disorder” is still a basis for rejection, Appellants submit that this generic term is definite.

This rejection should also be reversed.

#### **V. Claims 1, 12, 17, and 22 – self contradiction**

Claim 1 stands further rejected because the Examiner believes it is self-contradictory (Action, pages 8-9). Although the record is unclear, claims 12, 17, and 22 also appear to be rejected on the same basis (Action, page 9).

On the basis of his review of Stedman’s Medical Dictionary and the Merck Manual, the Examiner asserts that it is not possible to normalize the sleep/wake cycle without treating insomnia (Action, pages 8-9). Appellants disagree.

It appears that the basis of the rejection is that the Examiner equates an abnormal sleep/wake cycle with insomnia. As is apparent from Stedman’s and the Merck Manual (Evidence Appendix), insomnia, by definition, is an inability to sleep. Appellants have previously argued that an abnormal sleep/wake cycle can occur when a subject sleeps too much. Accordingly, insomnia is not synonymous with an abnormal sleep/wake cycle. Exclusion of treatment of insomnia from the method of claim 1 therefore is not self-contradictory.

In reply to these arguments, the Examiner has provided no reason why insomnia is synonymous with normalizing the sleep/wake cycle or why one skilled in the art would not be able to understand the metes and bounds of the present claims. Accordingly, there is no basis for the rejection, and it must be reversed.

With respect to claims 12 and 22, these claims are directed to various sleep disorders. As is well known in the art and discussed in the references provided by the Examiner, insomnia is not the only sleep disorder, nor is it an element of every sleep disorder. Accordingly, the exclusion of insomnia from the scope of claims 12 and 22 is not contradictory.

Claim 17 is directed to a method for increasing cognitive function in a sleep-deprived mammal not suffering from insomnia. As discussed, insomnia relates to an *inability* to sleep – not to lack of sleep per se. The conscious decision to stay awake is not synonymous with insomnia. Thus, a mammal may be deprived of sleep without suffering from insomnia. Accordingly, there is no contradiction in excluding mammals suffering from insomnia from the scope of claim 17.

#### **VI. Claims 1, 12, 17, and 22 - preambles**

Claims 1, 12, 17, and 22 stand rejected for indefiniteness because, in the Examiner's view, the results of the methods "are each insufficient to adequately define the particular disease condition being treated and thereby each noted term renders the associated claim incompletely defined." (Action, page 10) The Examiner further states that the claims "represent a therapeutic goal (a sleep-disorder-related symptom to be treated) but ... do not define with particularity a critical portion of the subject matter ... being claimed." (Action, page 10).

This rejection also appears to be based on the use of generic terms, and so is improper. A definite claim is one in which the scope is clear to the skilled artisan. In particular, there is no requirement for an applicant to claim treatment of a specific disorder rather than symptoms of a disorder. One skilled in the art would understand what is encompassed by "normalizing the sleep/wake cycle," "treating a sleep disorder," or "increasing cognitive function in a sleep-deprived mammal." Nothing more is required.

Regarding claim 1, the claim recites a method of normalizing the sleep/wake cycle. The term “normalize” means “to make conform to or reduce to a norm or standard” (Merriam-Webster Online Dictionary, Evidence Appendix). In addition, the term “sleep/wake cycle” is an art-known term denoting a mammal’s pattern of being awake and being asleep. Thus, “normalizing the sleep/wake cycle of a mammal” simply means making the sleep/wake pattern of a mammal conform to its norm or standard, the parameters of which are well known to practitioners in this field. Furthermore, some mammals are diurnal (i.e., active during the day), some are nocturnal (i.e., active during the night), and others are some combination of the two; thus, the norm or standard will depend on the individual mammal. One skilled in the art would easily be able to ascertain whether a mammal had an abnormal sleep/wake cycle, and claim 1 encompasses all methods of normalizing the sleep/wake cycle in mammals not suffering from insomnia. All of these things would be readily understood by one of skill in the art.

With respect to recitation of “sleep disorder” in claims 12 and 22, the term is defined in the specification on page 2, lines 27-28 and is also commonly used in the art, as evidenced by the enclosed PubMed search indicating that the term has appeared in over 500 references prior to the filing date of the present application (Evidence Appendix). One skilled in the art would be able to determine whether a mammal had a sleep disorder requiring treatment. Accordingly, one skilled in the art would understand the metes and bounds of the methods of claims 12 and 22.

Finally, claim 17 is directed to methods of increasing cognitive function in a sleep deprived mammal. “Cognitive function” is also an art-used term whose metes and bounds are apparent to one skilled in the art, as evidenced by a search of PubMed yielding 4563 references reciting the phrase prior to the December 20, 2002 priority date for the instant application (Evidence Appendix). Sleep deprivation is also well known in the art and requires no further explanation. Increasing cognitive function in a sleep deprived mammal would thus be understood by one of ordinary skill, and this claim is also definite.

The preamble of claims 1, 12, 17, and 22 are definite, and this rejection should be reversed.

### **VII. Claims 1-3, 5, 7-12, 22, and 26 – “in need thereof”**

The Examiner has rejected these claims because they do not provide a recitation of “in need thereof.” (Action, page 10). To address this rejection, Appellants amended claims 1, 12, and 22 to recite “in need thereof” in the amendment under 37 C.F.R. § 41.33 filed on June 16, 2009. Entry of the amendment renders this rejection moot.

### **VIII. Claims 17 and 20 – “problem sleepiness”**

The Examiner has requested clarification of these claims because “problem sleepiness” is not described in his medical dictionaries (Action, page 10). In reply, Appellants note that this term was previously misinterpreted by the Office during prosecution in the Action mailed on December 16, 2004. In reply, Appellants provided the Office with “Facts about Problem Sleepiness,” published in September 1997 by the National Institutes of Health with the Reply of June 16, 2005. Appellants again assert that “problem sleepiness” should be interpreted based on “Facts about Problem Sleepiness,” a copy of which is enclosed in the Evidence Appendix.

As clarification has been provided, the rejection is moot.

#### *Obviousness and Obviousness-type Double Patenting*

The final basis of appeal is the rejection of all claims for both obviousness and obviousness-type double patenting over Renshaw.

#### **I. The Claims Are Non-Obvious over Renshaw.**

The analytical framework to be used in determining obviousness was set forth by the U.S. Supreme Court in *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459 (1966):

Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background, the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or nonobviousness, these inquiries may have relevance.

The Supreme Court reaffirmed this standard for obviousness in *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 82 USPQ2d 1385 (2007).

In order to support a *prima facie* case of obviousness the Examiner must make appropriate findings of fact and consider these facts in light of evidence provided by the Appellant. Furthermore, any obviousness rejection requires an analysis of the differences between the prior art and the claimed invention, as well as reasoning why one skilled in the art would bridge the gap between the two (M.P.E.P. § 2141(III)). On this point, the Court states: “To facilitate review, this analysis should be made explicit. [s]ee *In re Kahn*, 441 F. 3d 977, 988 (CA Fed. 2006) (‘[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness’).” *KSR Int'l Co.*, 550 U.S. at 418, 82 USPQ2d at 1396.

For the present rejection, the Examiner has not properly considered the *Graham* factors. The entire basis of the rejection is:

The ‘703 reference [i.e., Renshaw] claims the “ameliorating a stimulant induced disorder,” wherein the “stimulant” is defined in claim 13 as “cocaine” and wherein the treatment comprises administration of a cytosine or cytidine compound including CDP-choline or CDP.

The claimed subject matter in the ‘703 reference [i.e., Renshaw] clearly is not identical with the subject matter in the instant application, but also clearly overlaps therewith in light of the common stimulant and common active ingredients, thereby rendering the instant claimed subject matter obvious. (Action, pages 12-13)

This position of the Examiner shows a lack of consideration of the scope of Renshaw, the scope of the present claims, and the differences between the two. The Examiner has also again made a general rejection of all claims without considering their individual limitations. Appellants are also unaware of any legal authority for the proposition that potential overlap alone is sufficient to establish a *prima facie* case of obviousness, and the Examiner provides no support for this position.

Proper consideration of the *Graham* factors indicates that the present claims are not obvious over Renshaw. With respect to scope and content, Renshaw is directed to methods of preventing or ameliorating a stimulant-induced disorder and cerebral vasoconstriction sequelae by administering a cytidine-containing or cytosine-containing compounds, such as CDP-choline (col. 1, line 53 – col. 2, line 10). Renshaw further states that cerebral vasoconstriction sequelae may include cognitive impairment (col. 3, ll. 22-29). The reference is completely silent with respect to disruptions of the sleep/wake cycle, any sleep disorder, and any effects of sleep deprivation.

In contrast to Renshaw, the present claims are directed to a method of normalizing the sleep/wake cycle of a mammal (claim 1); methods of treating a sleep disorder (claims 12 and 22), and a method for increasing cognitive function in a sleep-deprived mammal (claim 17). The claimed methods employ *inter alia* cytidine- or cytosine-containing compounds, such as CDP-choline. The sleep disorders treated may be caused by substance abuse disorders (e.g., claims 13, 14, 23, and 24) or not caused by substance abuse disorders (e.g., claim 19).

The significant differences between the prior art and the claimed invention are that Renshaw does not discuss sleep or sleep deprivation in any context, and there is nothing in Renshaw that connects stimulant use or cerebral vasoconstriction with disruptions in the sleep/wake cycle (claim 1), sleep disorders (claims 12 and 22), or cognitive impairment in sleep-deprived mammals (claim 17). Furthermore, claim 19 explicitly excludes treatment of a sleep disorder caused by substance abuse. Accordingly, Renshaw

does not overlap with claim 19, and the Examiner’s basis for the rejection is inapplicable to this claim.

The level of ordinary skill is discussed above with respect to the enablement rejection. While the methods of Renshaw are directed to treatment of individuals who may also be treated using the methods of claims 1, 12, 17, and 22, the Examiner has provided no rationale as to why this potential overlap in patient populations would lead the skilled artisan to modify the teachings of Renshaw to arrive at the methods of any of independent claims 1, 12, 17, or 22. Furthermore, each of these independent claims is directed to distinct subject matter, each requiring a separate rationale to support the rejection. While a physician or medicinal chemist could implement the distinct methods of claims 1, 12, 17, and 22 using the guidance provided by the present application, there is no reason why such an individual would alter the methods of Renshaw to produce what it claimed.

In sum, the Examiner has based the present rejection on a standard of “overlap” rather than consideration of the *Graham* factors. Proper consideration of the *Graham* factors shows that there is no connection between the teachings of Renshaw and the distinct methods of claims 1, 12, 17, or 22. Thus, there is no rationale to support the present rejection, and it should be reversed.

## **II. The Present Claims Are Not Obvious Variants of the Renshaw Claims**

In addition to the obviousness rejection over Renshaw, the Examiner has rejected the claims for obviousness-type double patenting over Renshaw. As stated in M.P.E.P. § 804(II)(B)(1), the analysis for a rejection for obviousness-type double patenting parallels that for a rejection under 35 U.S.C. § 103. In particular, the *Graham* factors must be considered in any obviousness-type double patenting rejection. A key difference between obviousness-type double patenting and obviousness is that for double patenting only the claims and those portions of the specification pertaining to the claimed invention are considered, while for obviousness the entire reference is considered (M.P.E.P.

§ 804(II)(B)(1)). It is axiomatic that any claim that is not obvious over an entire reference cannot be obvious over only a portion of the reference.

The nonobviousness of the distinct methods of claims 1, 12, 17, and 22 is discussed above. As noted, Renshaw is completely silent with respect to the sleep/wake cycle, sleep disorders, and impaired cognitive function in sleep-deprived subjects. In addition, the claims of Renshaw are focused on stimulant-induced conditions, i.e., stimulant-induced disorders in claim 1 and stimulant-induced cerebral vasoconstriction sequelae in claim 5. And the Examiner has not provided any reason, from the prior art, why one skilled in the art would apply a treatment for a stimulant-induced disorder or cerebral vasoconstriction sequelae to normalization of the sleep/wake cycle, treatment of a sleep disorder, or increasing the cognitive function of a sleep-deprived mammal. Accordingly, the rejection of the claims to each of these distinct methods should be reversed.

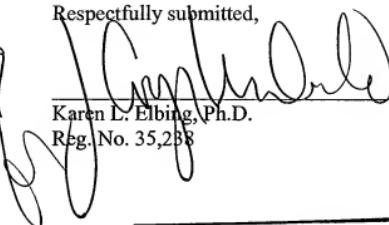
In addition, as discussed above, claim 19 is directed to treatment of a sleep disorder not caused by a substance abuse disorder. The only basis for this rejection is that the patient population of Renshaw overlaps with that of the present claims. As claim 19 explicitly excludes treatment of sleep disorders caused by substance abuse, there can be no overlap with the claims of Renshaw. Thus, the Office has failed to provide a basis to support the rejection of claim 19, and, for this reason as well, it should be reversed.

Conclusion

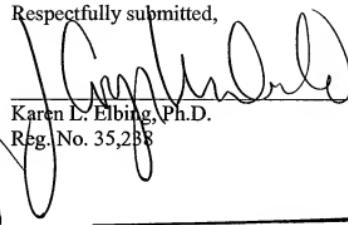
Appellant respectfully requests that the rejection of claims 1-3, 5, 7-20, and 22-26 be reversed. A petition to extend the period for filing a brief for five months was filed on June 16, 2009. If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Date: 6/22/09

Respectfully submitted,

  
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Claims Appendix –  
Claims after Amendment under 37 C.F.R. § 41.33

1. (Currently amended) A method of normalizing the sleep/wake cycle of a mammal in need thereof, said method comprising orally administering to said mammal a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal is not suffering from insomnia.
2. (Previously presented) The method of claim 1, wherein said administration reduces fatigue or tiredness, increases wakefulness, or improves the sleep quality of said mammal.
3. (Previously presented) The method of claim 1, wherein said compound is cytidine.
4. (Cancelled)
5. (Previously presented) The method of claim 1, wherein said compound is CDP-choline.
6. (Cancelled)
7. (Previously presented) The method of claim 1, wherein said compound is CDP.

8. (Previously presented) The method of claim 1, wherein said administration is chronic.

9. (Original) The method of claim 1, wherein said mammal is a human.

10. (Original) The method of claim 9, wherein said human is a child or adolescent.

11. (Original) The method of claim 9, wherein said human is an older adult.

12. (Currently amended) A method of treating a sleep disorder, said method comprising administering to a mammal in need thereof a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal's health is not compromised because of an existing physical condition and wherein said mammal is not suffering from insomnia.

13. (Original) The method of claim 12, wherein said sleep disorder is caused by a substance abuse disorder.

14. (Previously presented) The method of claim 13, wherein said substance abuse disorder is alcohol, caffeine, or cocaine dependence.

15. (Previously presented) The method of claim 12, wherein said sleep disorder is constructive or obstructive sleep apnea, restless leg syndrome, periodic limb movements, or narcolepsy.

16. (Previously presented) The method of claim 12, wherein said compound is CDP-choline.

17. (Currently amended) A method of increasing cognitive function in a sleep-deprived mammal, said method comprising administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) to a mammal suffering from sleep deprivation, wherein said mammal is not suffering from insomnia.

18. (Previously presented) The method of claim 17, wherein said compound is CDP-choline.

19. (Previously presented) The method of claim 12, wherein said sleep disorder is not caused by a substance abuse disorder.

20. (Previously presented) The method of claim 12, wherein said sleep disorder is problem sleepiness.

21. (Cancelled)

22. (Currently amended) A method of treating a sleep disorder, said method comprising administering to a mammal in need thereof a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, dipyridamole, propentofylline, or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), wherein said sleep disorder is not insomnia or sleep apnea.

23. (Previously presented) The method of claim 22, wherein said sleep disorder is caused by a substance abuse disorder.

24. (Previously presented) The method of claim 23, wherein said substance abuse disorder is alcohol, caffeine, or cocaine dependence.

25. (Previously presented) The method of claim 22, wherein said sleep disorder is restless leg syndrome, periodic limb movements, or narcolepsy.

26. (Previously presented) The method of claim 22, wherein said compound is CDP-choline.

27. – 30. (Cancelled)

Claims Appendix –  
Claims prior to Amendment under 37 C.F.R. § 41.33

1. (Previously presented) A method of normalizing the sleep/wake cycle of a mammal, said method comprising orally administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal is not suffering from insomnia.
2. (Previously presented) The method of claim 1, wherein said administration reduces fatigue or tiredness, increases wakefulness, or improves the sleep quality of said mammal.
3. (Previously presented) The method of claim 1, wherein said compound is cytidine.
4. (Cancelled)
5. (Previously presented) The method of claim 1, wherein said compound is CDP-choline.
6. (Cancelled)
7. (Previously presented) The method of claim 1, wherein said compound is CDP.

8. (Previously presented) The method of claim 1, wherein said administration is chronic.

9. (Original) The method of claim 1, wherein said mammal is a human.

10. (Original) The method of claim 9, wherein said human is a child or adolescent.

11. (Original) The method of claim 9, wherein said human is an older adult.

12. (Previously presented) A method of treating a sleep disorder, said method comprising administering to a mammal a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), or triacetyl uridine, wherein said mammal's health is not compromised because of an existing physical condition and wherein said mammal is not suffering from insomnia.

13. (Original) The method of claim 12, wherein said sleep disorder is caused by a substance abuse disorder.

14. (Previously presented) The method of claim 13, wherein said substance abuse disorder is alcohol, caffeine, or cocaine dependence.

15. (Previously presented) The method of claim 12, wherein said sleep disorder is constructive or obstructive sleep apnea, restless leg syndrome, periodic limb movements, or narcolepsy.

16. (Previously presented) The method of claim 12, wherein said compound is CDP-choline.

17. (Previously presented) A method of increasing cognitive function in a sleep-deprived mammal, said method comprising administering a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, propentofylline, or EHNA to a mammal suffering from sleep deprivation, wherein said mammal is not suffering from insomnia.

18. (Previously presented) The method of claim 17, wherein said compound is CDP-choline.

19. (Previously presented) The method of claim 12, wherein said sleep disorder is not caused by a substance abuse disorder.

20. (Previously presented) The method of claim 12, wherein said sleep disorder is problem sleepiness.

21. (Cancelled)

22. (Previously presented) A method of treating a sleep disorder, said method comprising administering to a mammal a therapeutically-effective amount of a compound comprising cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), deoxycytidine triphosphate (dCTP), cytidine diphosphate-choline (CDP-choline), cytosine, uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), triacetyl uridine, creatine, adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), S-adenosylmethionine, dipyridamole, propentofylline, or EHNA, wherein said sleep disorder is not insomnia or sleep apnea.

23. (Previously presented) The method of claim 22, wherein said sleep disorder is caused by a substance abuse disorder.

24. (Previously presented) The method of claim 23, wherein said substance abuse disorder is alcohol, caffeine, or cocaine dependence.

25. (Previously presented) The method of claim 22, wherein said sleep disorder is restless leg syndrome, periodic limb movements, or narcolepsy.

26. (Previously presented) The method of claim 22, wherein said compound is CDP-choline.

27. – 30. (Cancelled)

Evidence Appendix

- A. "Cognitive function" (PubMed search) – entered June 20, 2005
- B. "Facts about Problem Sleepiness" (NIH Publication No. 97-4071; September 1997) – entered June 20, 2005
- C. Fernandez (*Arzneimittelforschung. Drug Res.* 33:1073-1080 (1983)) – entered June 20, 2005
- D. Ferrer Internacional, S.A. (International Publication No. WO 01/72288) – entered June 27, 2005
- E. The Merck Manual, pages 1409-1414 – entered August 7, 2007
- F. "Normalize" (Merriam-Webster Dictionary) – entered June 20, 2005
- G. "Physical condition" (PubMed search) – entered November 3, 2006
- H. Radulovacki et al. (*J. Pharmacol. Exper. Ther.* 228:268-274 (1984)) – entered December 6, 2004
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B. "Facts about Problem Sleepiness" (NIH Publication No. 97-4071; September 1997) – entered June 20, 2005



# Problem Sleepiness

## WHAT IS PROBLEM SLEEPINESS?

Everyone feels sleepy at times. However, when sleepiness interferes with daily routines and activities, or reduces the ability to function, it is called "problem sleepiness." A person can be sleepy without realizing it. For example, a person may not feel sleepy during activities such as talking and listening to music at a party, but the same person can fall asleep while driving home afterward.

You may have problem sleepiness if you:

- consistently do not get enough sleep, or get poor quality sleep;
- fall asleep while driving;
- struggle to stay awake when inactive, such as when watching television or reading;
- have difficulty paying attention or concentrating at work, school, or home;
- have performance problems at work or school;
- are often told by others that you are sleepy;
- have difficulty remembering;
- have slowed responses;
- have difficulty controlling your emotions; or
- must take naps on most days.

## WHAT CAUSES PROBLEM SLEEPINESS?

Sleepiness can be due to the body's natural daily sleep-wake cycles, inadequate sleep, sleep disorders, or certain drugs.

### Sleep-Wake Cycle

Each day there are two periods when the body experiences a natural tendency toward sleepiness: during the late night hours (generally between midnight and 7 a.m.) and again during the midafternoon (generally between 1 p.m. and 4 p.m.). If people are awake during these times, they have a higher risk of falling asleep unintentionally, especially if they haven't been getting enough sleep.

### Inadequate Sleep

The amount of sleep needed each night varies among people. Each person needs a particular amount of sleep in order to be fully alert throughout the day. Research has shown that when healthy adults are allowed to sleep unrestricted, the average time slept is 8 to 8.5 hours. Some people need more than that to avoid problem sleepiness; others need less.

If a person does not get enough sleep, even on one night, a "sleep debt" begins to build and increases until enough sleep is





obtained. Problem sleepiness occurs as the debt accumulates. Many people do not get enough sleep during the work week and then sleep longer on the weekends or days off to reduce their sleep debt. If too much sleep has been lost, sleeping in on the weekend may not completely reverse the effects of not getting enough sleep during the week.

### Sleep Disorders

Sleep disorders such as sleep apnea, narcolepsy, restless legs syndrome, and insomnia can cause problem sleepiness. *Sleep apnea* is a serious disorder in which a person's breathing is interrupted during sleep, causing the individual to awaken many times during the night and experience problem sleepiness during the day. People with *narcolepsy* have excessive sleepiness during the day, even after sleeping enough at night. They may fall asleep at inappropriate times and places. *Restless legs syndrome (RLS)* causes a person to experience unpleasant sensations in the legs, often described as creeping, crawling, pulling, or painful. These sensations frequently occur in the evening, making it difficult for people with RLS to fall asleep, leading to problem sleepiness during the day. *Insomnia* is the perception of poor-quality sleep due to difficulty falling asleep, waking up during the night with difficulty returning to sleep, waking up too early in the morning, or unrefreshing sleep. Any of these sleep disorders can cause problem sleepiness. See page 4 for information on how to order fact sheets about the above sleep disorders.

### Medical Conditions/Drugs

Certain medical conditions and drugs, including prescription medications, can also disrupt sleep and cause problem sleepiness. Examples include:

- **Chronic illnesses such as asthma, congestive heart failure, rheumatoid arthritis, or any other chronically painful disorder;**
- **Some medications to treat high blood pressure, some heart medications, and asthma medications such as theophylline;**
- **Alcohol—Although some people use alcohol to help themselves fall asleep, it causes sleep disruption during the night, which can lead to problem sleepiness during the day. Alcohol is also a sedating drug that can, even in small amounts, make a sleepy person much more sleepy and at greater risk for car crashes and performance problems;**
- **Caffeine—Whether consumed in coffee, tea, soft drinks, or medications, caffeine makes it harder for many people to fall asleep and stay asleep. Caffeine stays in the body for about 3 to 7 hours, so even when taken earlier in the day it can cause problems with sleep at night; and**
- **Nicotine from cigarettes or a skin patch is a stimulant and makes it harder to fall asleep and stay asleep.**

### PROBLEM SLEEPINESS AND ADOLESCENTS

Many U.S. high school and college students have signs of problem sleepiness, such as:

- **difficulty getting up for school;**
- **falling asleep at school; and/or**
- **struggling to stay awake while doing homework.**

The need for sleep may be 9 hours or more per night as a person goes through adolescence. At the same time, many teens begin to show a preference for a later bed time, which may be due to a biological change. Teens tend to stay up later but have to get up early for school, resulting in their getting much less sleep than they need.

Many factors contribute to problem sleepiness in teens and young adults, but the main causes are not getting enough sleep and irregular sleep schedules. Some of the factors that influence adolescent sleep include:

- **social activities with peers that lead to later bedtimes;**
- **homework to be done in the evenings;**
- **early wake-up times due to early school start times;**
- **parents being less involved in setting and enforcing bedtimes; and**
- **employment, sports, or other extracurricular activities that decrease the time available for sleep.**

Teens and young adults who do not get enough sleep are at risk for problems such as:

- automobile crashes;
- poor performance in school and poor grades;
- depressed moods; and
- problems with peer and adult relationships.

Many adolescents have part-time jobs in addition to their classes and other activities. High school students who work more than 20 hours per week have more problem sleepiness and may use more caffeine, nicotine, and alcohol than those who work less than 20 hours per week or not at all.

## SHIFT WORK AND PROBLEM SLEEPINESS

About 20 million Americans (20 to 25 percent of workers) perform shift work. Most shift workers get less sleep over 24 hours than day workers. Sleep loss is greatest for night shift workers, those who work early morning shifts, and female shift workers with children at home. About 60 to 70 percent of shift workers have difficulty sleeping and/or problem sleepiness.

The human sleep-wake system is designed to prepare the body and mind for sleep at night and wakefulness during the day. These natural rhythms make it difficult to sleep during daylight hours and to stay awake during the night hours, even in people who are well rested. It is possible that the human body never

completely adjusts to nighttime activity and daytime sleep, even in those who work permanent night shifts.

In addition to the sleep-wake system, environmental factors can influence sleepiness in shift workers. Because our society is strongly day-oriented, shift workers who try to sleep during the day are often interrupted by noise, light, telephones, family members, and other distractions. In contrast, the nighttime sleep of day workers is largely protected by social customs that keep noises and interruptions to a minimum.

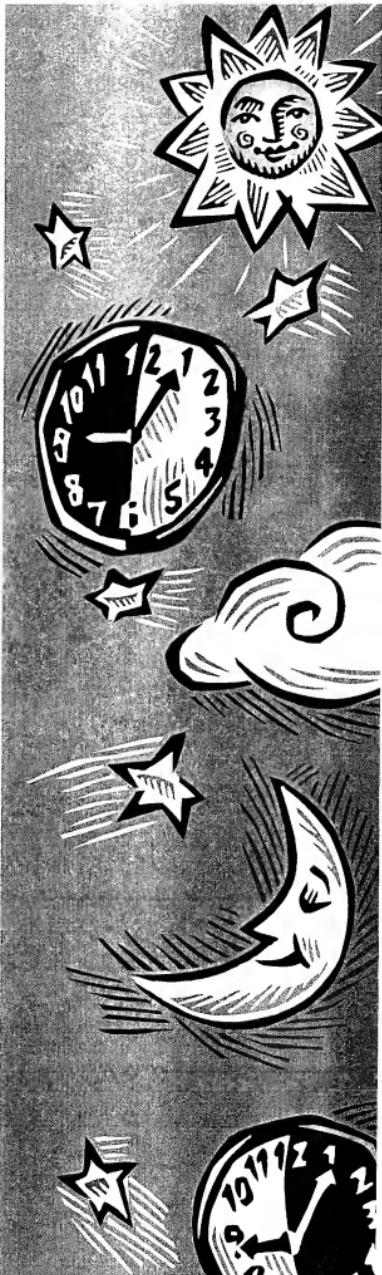
Problem sleepiness in shift workers may result in:

- increased risk for automobile crashes, especially while driving home after the night shift;
- decreased quality of life;
- decreased productivity (night work performance may be slower and less accurate than day performance); and/or
- increased risk of accidents and injuries at work.

## WHAT CAN HELP?

### Sleep—There Is No Substitute!

Many people simply do not allow enough time for sleep on a regular basis. A first step may be to evaluate daily activities and sleep-wake patterns to determine how much sleep is obtained. If you are consistently getting less than 8 hours of sleep per night, more sleep may be needed. A good approach is to gradually move to an earlier bed-



time. For example, if an extra hour of sleep is needed, try going to bed 15 minutes earlier each night for four nights and then keep the last bedtime. This method will increase the amount of time in bed without causing a sudden change in schedule. However, if work or family schedules do not permit the earlier bedtime, a 30- to 60-minute daily nap may help.

### Medications/Drugs

In general, medications do not help problem sleepiness, and some make it worse. *Caffeine* can reduce sleepiness and increase alertness, but only temporarily. It can also cause problem sleepiness to become worse by interrupting sleep.

While *alcohol* may shorten the time it takes to fall asleep, it can disrupt sleep later in the night, and therefore add to the problem sleepiness.

Medications may be prescribed for patients in certain situations. For example, the short-term use of sleeping pills has been shown to be helpful in patients diagnosed with acute insomnia. Long-term use of sleep medication is recommended only for the treatment of specific sleep disorders.

### If You're Sleepy—Don't Drive!

A person who is sleepy and drives is at high risk for an automobile crash. Planning ahead may help reduce that risk. For example, the following tips may help when planning a long distance car trip:

- **Get a good night's sleep before leaving.**
- **Avoid driving between midnight and 7 a.m.**
- **Change drivers often to allow for rest periods.**
- **Schedule frequent breaks.**

If you are a shift worker, the following may help:

- **decreasing the amount of night work;**
- **increasing the total amount of sleep by adding naps and lengthening the amount of time allotted for sleep;**
- **increasing the intensity of light at work;**
- **having a predictable schedule of night shifts;**
- **eliminating sound and light in the bedroom during daytime sleep;**
- **using caffeine (only during the first part of the shift) to promote alertness at night; or**
- **possibly using prescription sleeping pills to help daytime sleep on an occasional basis (check with your doctor).**

If you think you are getting enough sleep, but still feel sleepy during the day, check with your doctor to be sure your sleepiness is not due to a sleep disorder.

### WHERE TO GET MORE INFORMATION

For additional information on sleep and sleep disorders, contact the following offices of the National Heart, Lung, and Blood Institute of the National Institutes of Health:

- **National Center on Sleep Disorders Research (NCSDR)**

The NCSDR supports research, scientist training, dissemination of health information, and other activities on sleep and sleep disorders. The NCSDR also coordinates sleep research activities with other Federal agencies and with public and nonprofit organizations.

- **National Center on Sleep Disorders Research**
- **National Institutes of Health**
- **Two Rockledge Centre**
- **Suite 7024**
- **6701 Rockledge Drive, MSC 7920**
- **Bethesda, MD 20892-7920**
- **(301) 435-0199**
- **(301) 480-3451 (fax)**

- **National Heart, Lung, and Blood Institute Information Center**

The Information Center acquires, analyzes, promotes, maintains, and disseminates programmatic and educational information related to sleep and sleep disorders. Write for a list of available publications or to order additional copies of this fact sheet.

- **NHLBI Information Center**
- **P.O. Box 30105**
- **Bethesda, MD 20824-0105**
- **(301) 251-1222**
- **(301) 251-1223 (fax)**
- <http://www.nhlbi.nih.gov/nhlbi/nhlbi.htm>

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molecules), they do not release a larger number of molecules per unit time.

As a conclusion we may say that our study confirms this hypothesis and also confirms the results obtained in previous tests with similar determinations, with the advantage that our study showed a good tolerance of CDP-choline without troubles for the patients.

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## Efficacy and Safety of Oral CDP-Choline

### Drug surveillance study in 2817 cases

Monitored by R. Lozano Fernández

**Summary:** A drug surveillance study has been carried out with oral cytidine diphosphate choline (CDP-choline, citicoline, Somazin®) in 2817 patients of all ages, predominantly those between 60 and 80 years old. They were suffering from several diseases, mainly the vascular cerebral insufficiency and its evolution. Treatment was carried out for between 15 days and 2 months, the mean dose being 6 ml/d.

The efficacy of the treatment was determined on the basis of the disappearance, improvement or worsening of clinical manifestations, most frequently seen by patients. The most benefited clinical manifestations by the treatment were: dizziness disappearing in 43.4% of the cases, and improving in 25.3%; cephalgia disappearing in 46.5% and improving in 26.7%; insomnia with 38.6% and 24.1%, respectively, depression with 36.9% and 24.1% and memory shortage with 21.2% and 44.7% respectively. The best results were obtained in chronic cerebrovascular insufficiency, the improvements obtained in dizziness, cephalgia, insomnia, fatigue and speech troubles being the most important.

The safety of the drug was excellent since side effects were observed only in 3.01% of the patients. Among these effects, the most frequently seen were digestive troubles, observed in 3.6% of the cases.

**Zusammenfassung:** Wirksamkeit und Verträglichkeit von oral verabreichtem CDP-Cholin / Eine Studie an 2817 Patienten

Eine Studie mit oraler Verabreichung von Cytidindiphosphocholin (CDP-Cholin, Citicoline, Somazin®) wurde an 2817 Patienten jeden Alters durchgeführt. Patienten im Alter von 60 bis 80 Jahren waren am häufigsten vertreten. Sie zeigten verschiedene neurologische Prozesse, hauptsächlich vaskulär zerebrale Insuffizienz und verschiedene Formen der Altersrückbildung. Die Behandlungen dauerten 15 Tage bzw. 2 Monate bei einer mittleren Dosierung von 6 ml täglich.

Die Wirksamkeit der Behandlung wurde daran gemessen, ob die klinischen Manifestationen der Patienten verschwanden, sich verbesserten oder verschlechterten. Die meisten Patienten zeigten Schwindgefühle, die bei 48.4% ganz verschwanden und sich bei 25.2% wesentlich verbesserten. Patienten, die unter Schlaflosigkeit leidten, wurden zu 38.6% geholfen und 24.9% fühlten sich wesentlich besser. Patienten mit Kopfschmerzen waren zu 46.3% vollkommen geholfen und zu 26.7% wesentlich verbessert. Depressionen verschwanden bei 36.9% und bei 24.1% waren sie verbessert. Gedächtnisschwäche war bei 21.2% verschwunden und bei 44.7% verbessert. Die besten Ergebnisse wurden in Fällen von chronischer zerebrovaskulärer Insuffizienz erzielt. Die Er-

gebnisse, die bei Schwindelgefühl, Cephalgie, Schlaflosigkeit und Sprachschwierigkeiten erreicht wurden, sind von nicht geringerer Bedeutung.

Die Verträglichkeit kann als optimal bezeichnet werden, weil sich nur bei 5,01% der Patienten unerwünschte Nebenwirkungen zeigten. Diese bestanden hauptsächlich in Magenbeschwerden, die in 3,6% der behandelten Fälle registriert wurden.

Resumen: Eficacia y Seguridad de CDP-colina por vía oral / Estudio de Farmaco vigilancia sobre 2817 casos

Se ha llevado a cabo un estudio de farmacovigilancia con cíticoline disfatoato de colina (CDP-colina, citocolina, Somazina®) oral en 2817 pacientes de todas las edades, con predominio de los comprendidos entre los 60 y los 80 años. Se hallaron efectos de diversos procesos neurológicos, predominando la insuficiencia vasculocerebral y la involución senil. La duración del tratamiento fue de 15 días a 2 meses, siendo la dosis media administrada la de 6 ml al día.

La eficacia del tratamiento se valoró en base a la desaparición, mejoría o empeoramiento de las manifestaciones clínicas más frecuentes presentadas por los pacientes. Las manifestaciones clínicas más beneficiadas por el tratamiento fueron: el vértigo, que desapareció en el 41,4% de los casos y mejoró en el 25,7%; la cefalea, que desapareció en el 46,5% y mejoró en el 26,7%; el trastorno, con el 30,6% y el 24,9% respectivamente; la depresión, con el 26,9% y el 24,1%; y el déficit de memoria, con el 21,4% y el 44,7% respectivamente. Los mejores resultados se observaron en la insuficiencia cerebrovascular crónica, destacando las mejorías obtenidas en el vértigo, la cefalea, el insomnio, la fatiga y los trastornos del sueño.

La tolerancia a la medicación fue excelente, pues sólo se constataron efectos colaterales en el 5,01% de los pacientes. Entre estos efectos, los más frecuentes fueron los digestivos, constatados en el 3,6% de los casos.

Key words: CDP-Choline · Cerebrally active agents · Cerebrovascular disorders · Cito coline, clinical studies, efficacy, safety · Cytidine diphosphate choline · Senile involution · Somazina®

## 1. Introduction

The characteristics of aging phenomena: universality and progressivity are going to influence all the regulation mechanisms in aged people and the reactivity of their organism, always closer to pathological situations than in people of other age.

Therefore, while going through the 3rd age, there is a reduction in adaptation capacity and an increase in the vulnerability against certain circumstances.

The functional modifications may be considered in their whole aspects or by systems of organs.

The behaviour of the nervous system has a special characteristic due to its close relationship with arterial regulation.

Brain decreases in size and weight (this last one till 10%). The encephalic atrophy is more marked in the frontal area; the circumscriptions become reduced and the size of the grooves increases.

The change in colour of white substance is characteristic; it becomes darker by pigment accumulation. The most frequent pigments are the lipofuscin and amyloidaceous bodies, similar to starch grains in astrocytic prolongations.

There is a lot of neuronal changes, with years there appear inclusion bodies (Ley body) observing also banks of neurofibers. But one of the most significative signs are the so named senile plates, very frequent in the interstitial tissue.

Parallel to the morphological changes, there are also changes in the biochemical composition, specially in the constitution of structural phospholipids that compose the neuronal membranes and are responsible for the neurophysiological interchanges.

The scope of this work is to collect the objective and subjective modifications as to the behaviour and symptomatology of the neurological processes, accompanying the changes in the 3rd age, observed after the administration of cytidine diphosphate choline (CDP-choline, cito coline, Somazina®).

\*) Manufacturer: Ferrer Internacional S.A., Barcelona (Spain).

## 1.1. Scope

When a drug has shown an efficacy and safety, sufficient to be introduced in clinical therapy, it is necessary to go on with the studies in a high number of cases as to determine, as clearly as possible, all the indications and determine also the incidence of possible side-effects.

This is the scope of this study with cito coline, a drug which during the last 5 years has shown its clinical efficacy and safety, on parenteral as well as on oral administration. This last route of administration has been tested in patients with cerebrovascular insufficiency, sequelae from cerebral vascular accident, involutive cerebral deterioration (senile involution, Parkinson's syndrome, depressive syndrome and learning troubles; in all these indications cito coline has shown its beneficial effects.

## 2. Material and methods

### 2.1. Participating physicians

The study was carried out with 382 Spanish physicians, with the following geographical distribution:

Catalonia/Balearic islands	118
Castile	50
Asturias	80
North	52
Centre/Extremadura	57
Northeast	23
Canary islands	2
Total	382

## 2.2. Patient population

This study included 3,000 cases; only 2,817 patients suffering from several neurological processes have been tabulated.

The sex distribution was as follows: 1,492 men (53,3%) and 1,306 women (46,7%). In 19 of the cases there is no sex specification.

The age/sex distribution by age and sex is shown in Fig. 1, where it can be seen that the highest percentage is between 60 and 80 years, with a homogeneous distribution between both sexes. Considering that the most important diagnoses, as we shall see later on, are those related to chronic cerebrovascular insufficiency and senile involution, this percentage is predominant in ages over 60 years.

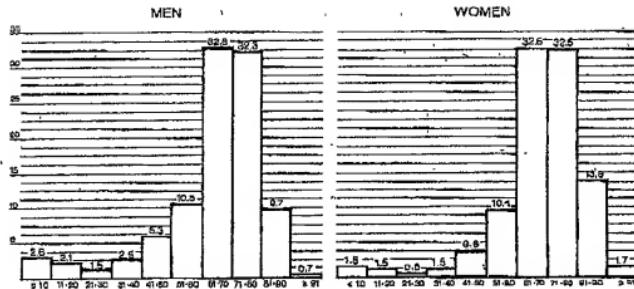


Fig. 1: Distribution by age and sex.

### 2.3. Diagnosis

Neurological diagnosis are included in Table 1, cerebrovascular insufficiency and senile involution being the most important components (2,278).

17.3% (489 cases) of the 2,817 patients showed more than one diagnosis of those indicated in Table 1; 10.2% (285 cases) were classed by the researchers of cerebrovascular insufficiency associated with senile involution.

Table 2 shows the per centual distribution of ages according to the diagnosis.

In chronic cerebrovascular insufficiency, senile involution and cerebro-vascular accident, the higher percentages correspond to the more advanced ages. 88.2% of the cases with chronic cerebrovascular insufficiency were more than 60 years old, percentage increased to 95.7% in senile involution. On the contrary, the sequelae of cranial traumas is important among those younger than 40 years, being in a direct relationship with their physical activity.

The distribution by sex and diagnosis is very homogeneous, except in the sequelae of cerebral traumas. In these cases, 68.2% of our sample were men, compared to 31.8% of women (See Table 3).

Table 1: Diagnosis distribution.

Diagnosis	No. of cases
Chronic cerebrovascular insufficiency	1,240
Senile involution	1,038
Cerebral vascular accident sequelae	524
Sequelae of cerebral traumatis	411
Other	272

Table 2: Percentage distribution of ages according to diagnosis.

Diagnosis	< 40 yrs.	41-60 yrs.	61-70 yrs.	> 70 yrs.
Chronic cerebrovascular insufficiency	0.7	11.1	35.7	52.5
Senile involution	0.3	4.3	31.2	64.2
Sequelae of cerebral-vascular accident	1.2	19.0	33.8	40.1
Sequelae of cerebral traumatis	41.3	38.1	12.2	8.4
Other	30.9	25.5	19.1	24.5

Table 3: Diagnosis distribution by sex.

Diagnosis	Mean	Women
Chronic cerebrovascular insufficiency	(No.) 659	374
(%) 23.4	46.6	
Senile involution	(No.) 490	544
(%) 47.4	52.6	
Sequelae of cerebral-vascular accident	(No.) 293	220
(%) 57.3	42.7	
Sequelae of cranial traumatis	(No.) 122	57
(%) 68.2	31.8	
Other	(No.) 192	176
(%) 51.9	48.1	

### 2.4. Associated pathology

Among the 2,817 patients, there was in 1,423 of them another associated non-neurological pathology. Fig. 2 shows the distribution of concomitant diseases related with every specific diagnosis previously mentioned.

Hypertension and diabetes have been specially considered as diseases with a high neurological risk.

In chronic cerebrovascular insufficiency, 20.4% of the patients were diagnosed from an associated arterial hypertension and 17.8% from diabetes. 76.1% were carriers of another non-neurological disease, specially arthrosis, depression and gastroduodenal ulcers.

309 out of the 1,038 cases of senile involution showed another associated pathology; 20.6% were diabetic and 19.5% hypertensive patients.

In patients affected with sequelae of cerebral-vascular accident, 272 cases were carriers of an associated pathology; hypertension in 36.5% being predominant; diabetes represented 21.2%.

In the sequelae of cranial traumas, 141 cases of diabetes and another of hypertension were observed. 14 out of the 32 reported cases were patients aged between 41 and 60 years.

### 2.5. Treatment period

The observation period was extended to 60 days, the controls being performed at 0, 15, 30 and 60 days.

If the symptomatology disappeared in any of these observation periods, then this date was considered as the end of the treatment. Treatment period is detailed in Table 4, according to its end date after 15, 30 or 60 days.

As it may be seen, most of the cases, namely 2,104 (78.5%), belongs to the end period after 60 days.

Table 4: Treatment period.

Days	No. of patients	%
15	169	6.3
30	405	15.2
60	2,104	78.5
Total	2,681	100

### 2.6. Dosage

Patients were administered  $5.96 \pm 0.048$  ml (average  $\pm$  SD) by oral route.

Each ml is equivalent to 100 mg of citochine.

In Fig. 3 the percentage of patients according to the dose for each diagnostic group is expressed.

In chronic cerebrovascular insufficiency 61.2% of the sample was treated with 6 ml/day and 15.5% with higher doses. In senile involution, 59.6% of the patients received 6 ml/day and 15.9% more than 6 ml/day. The highest percentage (65.3%) of the dose of 6 ml appears in the cases of cerebral-vascular accident sequelae, 17% of whom received more than 6 ml. In the sequelae of cerebral traumas, 6 ml were administered in 60.6% and more than 6 ml in 11.1% of the cases.

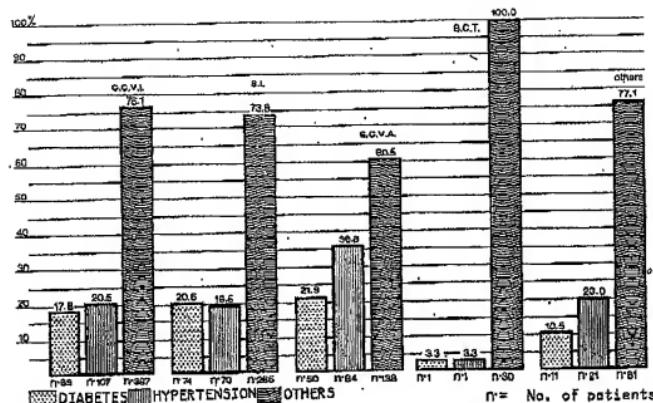


Fig. 2: Associated pathology. For abbreviations of Table 5.

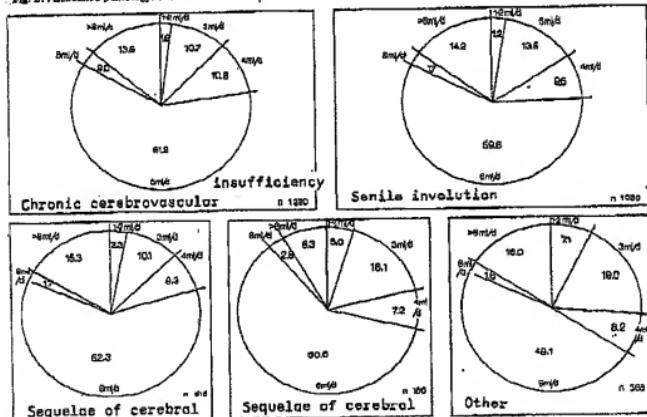


Fig. 3: Percentage of patients according to dosage (mg/day).

#### 2.7. Concomitant treatments

Considering that there was another associated pathology in 1,423 cases, as previously mentioned, the treatment of these diseases was maintained, if this was regarded necessary in the evolution of their process.

In Table 5 the data related to the concomitant treatment received are detailed, including the number, percentage and characteristics of the treatment grouped within the etiologic diagnosis.

Considering that there were patients with more than one diagnosis of disease, the treatments were more than one drug, the number of concomitant treatments is 1,930, with a predominance in chronic cerebro-vascular insufficiency, with 720 treatments (47%). As a ther-

apy, vasodilator drugs and other treatments for cardiovascular therapy, with 537 and 549 treatments, respectively, were the most frequent therapies.

#### 2.8. Studied parameters

The results of the treatment were evaluated according to the modifications observed in the intensity of the signs and symptoms:

1. dizziness;
2. cephalgia;
3. fatigue;
4. insomnia;
5. speech troubles;

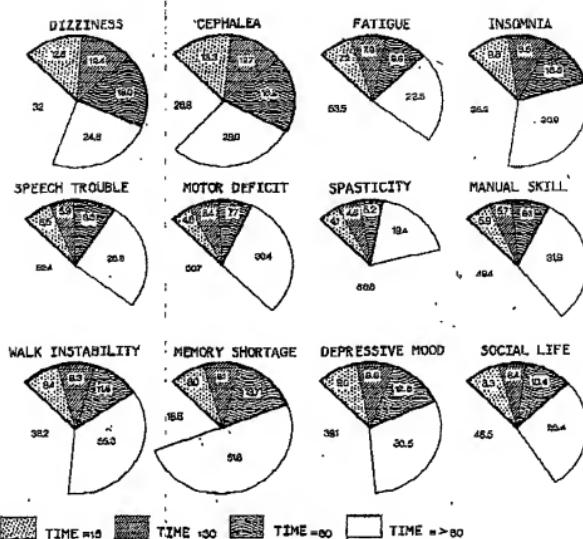


Fig. 4: Whole percentage of symptom disappearance in each control related to the total of patients. For all of them:  $p < 0.001$ .

6. motor deficits;
7. spasticity;
8. manual skill;
9. walk instability;
10. memory shortage;
11. depressive mood;
12. social life;

Each symptom manifestation is evaluated from 0 to 3 at the beginning and at the end of the treatment according to the following criteria:  
 0 = lack of symptomatology;  
 1 = slight;  
 2 = moderate;  
 3 = severe.

#### 2.9. Complementary tests

In order to check the diagnosis with an objective test and to see the drug's effect, an electroencephalogram was performed in 329 (12%) patients at the beginning and at the end of the treatment.

In 99 (35.2%) patients, a complete analytical study was carried out as to control any possible influence of citicoline on the hematopoietic, hepatic and renal functions.

#### 3. Results

##### 3.1. Evaluation

The analysis of the results was performed with the SPSS statistical Package of Chicago University (USA).

With this program, the evolution of the 12 signs and symptoms previously detailed in the paragraph "Parameters" have been analyzed, studying the number and percentage of disappearances, improvements, maintenances and worsenings of each symptom/sign, expressing the results individually for each parameter in function of the following variables:

Number of symptomatological disappearances. Whole percentage of the symptomatological disappearances in time, days 15, 30 and 60.

Percentage of symptomatological disappearances by etiological diagnosis in the different controls.

Number and percentage of improvements and worsenings. Number and percentage of symptomatological improvements according to the etiological diagnosis.

#### 3.2. Number of symptomatological disappearances

The number of patients showing at the beginning the 12 signs and symptoms have been studied; that is to say, those with total disappearance of their symptomatology.

The results are shown in Table 6, where the percentage of symptomatological disappearances is also shown in connection with the number of patients showing this symptomatology at the beginning.

Table 5: No. of patients receiving concomitant treatments.

Diagnosis <sup>a</sup>	No. of patients	Antihypertensives	Vasodilators	Other cardiovascular drugs	Anxiolytics	Other
C.C.V.I.	120(47.7)	146	197	210	33	442
S.I.	56(21.2)	103	164	144	45	556
S.C.V.A.	32(12.0)	98	96	142	22	157
S.C.T.	8(3.5)	2	18	7	12	70
Other	236(91.3)	93	62	46	20	179

<sup>a</sup> There were patients with more than one diagnosis.

In parentheses: %  
 C.C.V.I. = Chronic cerebrovascular insufficiency; S.I. = senile involution; S.C.V.A. = sequelae of cerebral-vascular accident; S.C.T. = sequelae of cerebral trauma.

In our sample, the most predominant symptom is the memory shortage with 2,249 cases of 2,817; thereafter cephalgia with 2,027, dizziness with 1,874, walk instability with 1,754, insomnia with 1,742 and depression with 1,667.

Dizziness is the symptom with a greater percentage of disappearance on the initial value with 48.4% of normalization of the equilibrium.

Thereafter the cephalgia with 46.5% of disappearance and fatigue with 39.5%.

### 3.3. Percentage of symptomatological disappearances as a function of time

Considering that the total disappearance of signs and symptoms in the evolution of a disease is the most objective parameter to evaluate the therapeutic effect of a drug, the percentage of disappearances in each observation period of the patient, in the days 15, 30 and 60 after the therapy with oral atropine, has been studied.

Fig. 4 shows for each sign and symptom the different percentages on the total of the sample (2,817 patients) of the disappearances of the symptomatology in each control and its persistency at the end of the treatment after 60 days.

Numbers shown in the figure correspond to the percentage of patients not showing this symptom at the beginning of the evaluation. The dotted space is the percentage of disappearances after 15 days, the dashed space is the percentage of disappearances after 30 days, the waved space after 60 and the white space the percentage of persistencies which, as we shall see later, may correspond to improvements.

The greatest percentages at the beginning of the treatment correlate, of course, with those shown in 3.2. Memory shortage, cephalgia, dizziness, walk instability, insomnia and depression, in this order, are the predominant symptoms.

#### After 15 days of observation

The highest percentages, on the total sample of 2,817 patients, of disappearances correspond to cephalgia with 13.3%, dizziness with 12.7%, insomnia with 9.8%, depression with 9%, walk instability with 8.4%, social life with 8.3% and memory shortage with 8%.

#### After 30 days

The percentage follows a very similar line: cephalgia disappeared in another 12.7%, dizziness in 12.4%, insomnia in 9.5%, depression in 8.8%, walk instability in 8.3% and memory shortage in 8.1%.

#### At the end of treatment

The following percentages were found as an addition of the previous ones: cephalgia 16.2%; dizziness 18%; memory shortage 13.4%; insomnia 13.5%; depression 12.8%; and walk instability 11.8%.

After the addition of all the percentages after 60 days, the order in importance is as follows:

dizziness 43%;  
cephalgie 34%;  
insomnia 33%;  
depression 31%;  
memory shortage 30%;  
obtaining in these symptoms the normalization after 60 days.

### 3.4. Percentage of symptomatological disappearances by etiological diagnosis in the different controls

Fig. 5, 6, 7 and 8 show the different disappearance percentages within each diagnosis group, in the different controls of each sign or symptom.

In chronic cerebrovascular insufficiency, the first symptoms to disappear are dizziness, cephalgia, insomnia, fatigue and speech trouble.

In senile involution: cephalgia, dizziness and speech troubles. In the sequelae of cerebral accident, cephalgia and dizziness especially.

In posttraumatic sequelae, the social life troubles, depression and fatigue.

A similar trend was observed in the percentages reached at the end of the two-month observation period.

#### Chronic cerebrovascular insufficiency

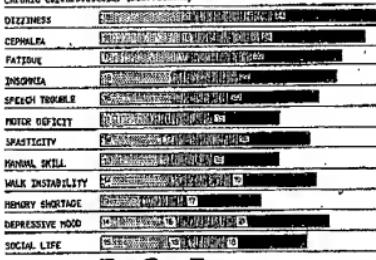


Fig. 5: Percentage of symptom disappearance according to treatment period. Chronic cerebrovascular insufficiency.

#### Senile involution

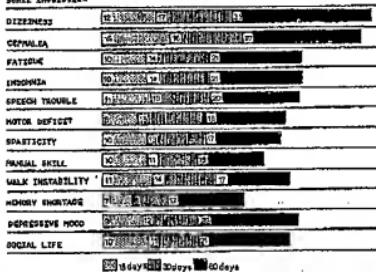


Fig. 6: Percentage of symptom disappearance according to treatment period. Senile involution.

#### Sequelae of cerebral vascular accident

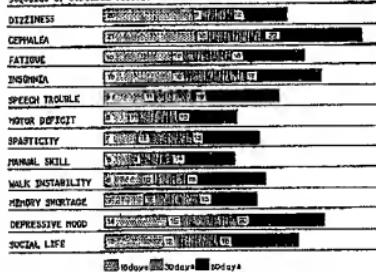


Fig. 7: Percentage of symptom disappearance according to treatment period. Sequelae of cerebral vascular accident.

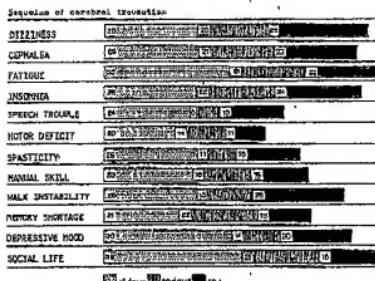


Fig. 8: Percentage of symptom disappearance according to treatment period. Sequela of cerebral traumatisms.

Table 6: Number of symptomatological disappearances.

Symptom	No. of patients showing the sign	No. of patients with sign disappearance	% of disappearance related to the initial numbers
Dizziness	1,874	907	48.4
Cephalgia	2,027	943	46.5
Fatigue	1,265	499	39.5
Insomnia	1,424	679	48.0
Speech trouble	1,020	451	35.0
Motor deficits	1,622	561	26.5
Spasticity	859	276	31.0
Manual skill	1,405	385	27.3
Walk instability	1,270	370	25.0
Memory shortage	1,249	474	37.1
Depressive mood	1,667	615	36.0
Social life	1,480	518	35.0

All the studied parameters have shown highly significant percentage ( $p < 0.001$ ) of healings.

Table 7: Number and whole percentage of improvements and worsenings in signs and symptoms at the end of treatment.

Symptoms	Improvement		Worsening	
	N	%	N	%
Dizziness	711	25.2	43	1.5
Cephalgia	732	26.7	41	1.5
Fatigue	424	15.1	45	1.6
Insomnia	707	24.0	37	1.2
Speech troubles	482	17.1	33	1.2
Motor deficit	565	20.1	33	1.2
Spasticity	320	11.4	50	1.8
Manual skill	541	19.2	59	2.1
Walk instability	634	22.4	53	1.8
Memory shortage	1,259	44.7	56	2.0
Depressive mood	680	24.1	48	1.7
Social life	541	19.3	73	2.6

Table 8: Number and percentage of improvements in signs and symptoms according to the diagnosis.

	Dizziness	Cephalgia	Fatigue	Insomnia	Speech troubles	Motor deficit	Spasticity	Manual skill	Walk instability	Memory deficit	Depressive mood	Social life
C.C.V.I.	N %	379 53.6	363 48.4	200 47.4	311 44.4	180 37.5	205 36.4	120 37.9	200 37.1	291 42.5	612 50.5	298 44.0
Spinal involvement	N %	258 36.5	275 56.7	198 46.0	325 48.3	173 34.6	193 34.3	108 34.1	221 41.0	222 41.2	550 42.9	221 47.4
Sequelae vascular accident	N %	110 15.6	169 36.7	76 17.6	117 16.7	202 42.1	237 42.1	142 44.8	202 37.5	208 30.4	222 17.7	125 18.2
Traumatism sequelae	N %	54 7.6	74 9.9	14 3.3	33 4.7	24 5.4	27 4.8	16 3.0	15 2.8	22 3.2	30 4.3	12 3.2

C.C.V.I.: see Table 5.

### Analytic

The counts of erythrocytes, leukocytes, glucose, uremia, creatinine, transaminases have not shown significant changes.

### 3.7. Safety

In general, the safety of oral citicoline was excellent, without any discontinuation of treatment due to side-effects.

151 side-effects have been observed, corresponding to digestive symptoms (nausea, gastralgia, diarrhoea) in 102 cases and vascular symptoms in 16 cases.

Digestive 102;  
vascular 16;

other 33. The vascular effects correspond to the reported cases of hypotension and cardiac rhythm troubles (bradycardia or tachycardia).

In 94.99% of patients, no side-effects were observed.

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(54) Title: USE OF CDP-CHOLINE FOR THE TREATMENT OF ALCOHOL WITHDRAWAL SYNDROME

(57) Abstract: The invention relates to the use of CDP-Choline or its pharmaceutically acceptable salts for the preparation of a medicinal product for the treatment of alcohol withdrawal syndrome at daily doses equivalent to 0.5-2g of free CDP-Choline.

USE OF CDP-CHOLINE FOR THE TREATMENT OF ALCOHOL WITHDRAWAL SYNDROME

DESCRIPTION

The present invention relates to the use of CDP-choline for the treatment of alcohol withdrawal syndrome.

The toxic effects of alcohol on central nervous system are basically exerted on neuronal membrane and synapses (Leonard B. E., *Alcohol Alcohol.*, 1986: 21(4), 325-338).

Histological alterations of neuronal structure consist in a lesser branching of hippocampus nerve cells and Purkinje's cells. Comparison of brains from healthy subjects with those from alcoholic patients revealed a lesser branching of pyramidal neuronal basal dendrites in upper cerebral cortex and motor cortex (Ledig M. and Mandel P., *M S-Medecine Sciences*, 1988: 4(6), 352-357).

Chronic alcohol abuse has also been reported to impair dopamine receptor sensitivity. This effect is probably related to changes in neuronal membrane fluidity and in the number and functionality of receptors, as well as to a decrease in acetylcholine reuptake and dopamine deficiency (Carlen P. L. and col., *Ann. Neurol.*, 1981: 9(1), 84-86).

CDP-choline (cytidine diphosphate choline, Citicoline) is a key intermediate in the synthesis of structural phospholipids present in the neuronal membrane (Kennedy E. P. and Weiss S. B., *J. Biol. Chem.*, 1956; 222, 193-214) and plays an important role in its formation and repair when the phospholipidic structure is damaged by endogenous or exogenous causes involving a decrease in cytidine and choline uptake.

The administration of CDP-choline enhances dopamine synthesis and release (Martinet M. et al., *Biochem. Pharmacol.*, 1981: 30(5), 539-541) as well as choline and acetylcholine brain levels. The administration of repeated doses of CDP-choline produces an increase of brain phospholipid levels, which is secondary to an increase of cytidine and choline plasma levels (Agut J. et al., *Ann. New York Acad. Sci.*, 1993: 695, 318-320).

Surprisingly, the applicants have found out that the administration of CDP-choline to alcoholic patients reduces the duration and intensity of their withdrawal symptoms and induces an evident recovery in a significant proportion of patients.

The use of CDP-choline according to the present invention, which includes a method for treating alcohol withdrawal syndrome, comprises the administration of an effective

amount of CDP-choline or a pharmaceutically acceptable salt thereof to an alcoholic patient.

According to the present invention, CDP-choline is administered as free compound or as a pharmaceutically acceptable salt, whether in anhydrous or hydrated form, conveniently mixed with pharmaceutical carriers and/or excipients, to humans at daily doses of 0.5 to 2 g inclusive in free CDP-choline, preferably from 0.5 to 1 g inclusive, both orally and parentally. Pharmaceutically acceptable salts of CDP-choline include its alkaline or alkaline earth salts, such as its sodium, potassium, calcium and magnesium salts or its acid addition salts with a mineral or organic acid, such as hydrochloric acid, hydrobromic acid, sulphuric acid, phosphoric acid, acetic acid, trifluoroacetic acid, citric acid, lactic acid, malonic acid, tartaric acid, acrylic acid, metacrylic acid, malic acid, maleic acid, fumaric acid, benzoic acid, salicylic acid, cinnamic acid, methane sulphonic acid, benzenesulphonic acid, p-toluensulphonic acid and nicotinic acid.

CDP-choline and its salts, whether as anhydrous or hydrated substances, under the invention may be administered orally in the form of tablets, capsules, powder, granules, cachets, lozenges, solution, suspension, emulsion, syrup, gel and the like; or parenterally in the form of solution,

suspension, emulsion or the like for intravenous or intramuscular injection.

EXAMPLES

The present invention is illustrated by the Examples that follow. Those skilled in the art will be able to make any change provided the specific embodiment of the invention is not modified and, therefore, the invention is not limited to the specific details of the Examples.

EXAMPLE 1: 500 mg tablets

CDP-choline, sodium salt	522.5 mg
Talc	30.0 mg
Magnesium stearate	3.0 mg
Silicon dioxide	2.5 mg
Croscarmellose sodium	20.0 mg
Corn starch	20.0 mg
Microcrystalline cellulose s.q.	780.0 mg

EXAMPLE: 25% oral solution

CDP-choline, sodium salt	26.12 g
70% Sorbitol	20.00 g
Methyl p-hydroxybenzoate	0.16 g

Propyl p-hydroxybenzoate	0.04 g
Disodium citrate	0.60 g
Saccharin sodium	0.02 g
Strawberry essence	0.04 g
Red Punzo 4R	0.50 mg
Anhydrous citric acid	0.05 g
Purified water s.q.	100.00 ml

EXAMPLE 3: Solution for injection

CDP-choline, sodium salt	522.50 mg
Hydrochloric acid, pH 6.0-6.5 s.q.	
Water for injection s.q.	4.00 ml

EXAMPLE 4 : Open clinical study of CDP-choline in alcohol withdrawal syndrome

The progress of alcohol withdrawal syndrome was assessed in an open study conducted in 197 patients. CDP-choline was administered at doses of 500 mg/d i.m. or 600 mg/d p.o. for 60 days. At 30 and 60 days following treatment, significant differences ( $p<0.001$ ) were observed in the assessments performed. At 60 days, 55.83% of patients had given up drinking alcohol and 31.97% of patients drank much less. A significant improvement was observed on anxiety, tremor, disorientation, insomnia, dysarthria, tendency to suicide and neuritic pains.

EXAMPLE 5: Open, randomized, comparative clinical study of CDP-choline in alcohol withdrawal syndrome versus clomethiazole and vitamin B.

An open, randomized and comparative study on the conventional therapy of alcohol withdrawal syndrome was conducted in 40 patients. Patients were randomly distributed in two groups of 20. One of the groups was used as control and received clomethiazole and vitamin B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub>. This treatment regimen was maintained for 8 days, and then patients were given diazepam until completion of treatment (60 days). The other group of patients received the same treatment regimen plus CDP-choline 500 mg i.m. every 12 h for the first 30 days and CDP-choline 200 mg i.m. every 8 h for the remaining 30 days. The patients who received CDP-choline plus the conventional therapy showed significant differences versus control at 30 days following treatment in tremor incidence ( $p<0.05$ ), cramps ( $p<0.05$ ), asthenia ( $p<0.05$ ), emotional lability ( $p<0.01$ ), nervousness ( $p<0.05$ ) and social withdrawal ( $p<0.05$ ).

CLAIMS

1. The use of CDP-choline or of a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of alcohol withdrawal syndrome.

2. The use according to claim 1 in which the pharmaceutically acceptable salts of CDP-choline are its alkaline or alkaline earth salts or its salts with mineral or organic acids such as hydrochloric acid, hydrobromic acid, sulphuric acid, phosphoric acid, acetic acid, trifluoroacetic acid, citric acid, lactic acid, malonic acid, tartaric acid, acrylic acid, metacrylic acid, maleic acid, maleic acid, fumaric acid, benzoic acid, salicylic acid, cinnamic acid, methane sulphonic acid, benzenesulphonic acid, p-toluenesulphonic acid and nicotinic acid, in anhydrous or hydrated form.

3. The use according to claims 1 or 2 at daily dose in equivalent amounts of free CDP-choline ranging from 0.1 to 2 g.

4. The use according to claim 3, wherein the dose range from 0.5 to 1 g.

5. A method for the treatment of alcohol withdrawal syndrome comprising administering to an alcoholic patient in need thereof an effective amount of CDP-choline or of a pharmaceutically acceptable salt thereof.

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(54) Title: USE OF CDP-CHOLINE FOR THE TREATMENT OF ALCOHOL WITHDRAWAL SYNDROME

(57) Abstract: The invention relates to the use of CDP-Choline or its pharmaceutically acceptable salts for the preparation of a medicinal product for the treatment of alcohol withdrawal syndrome at daily doses equivalent to 0.5-2g of free CDP-Choline.

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International Application No

PCT/EP 01/03536

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According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**Minimum documentation searched (classification system followed by classification symbols)  
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 07385 A (LUKAS SCOTT ; RENSHAW PERRY F (US); MCLEAN HOSPITAL CORP (US)) 18 February 1999 (1999-02-18) *cf. abstract, page 2, lines 10-21, page 4, lines 4-8, page 10, table 1*	1-5
Y	TORNOS M.E., ET AL.: "Effect of oral CDP-choline on experimental withdrawal syndrome" ARZNEIMITTEL-FORSCHUNG, vol. 33, no. 7a, 1983, pages 1018-11021, XP001026424 *cf. summary, introduction, page 1021, left col., "conclusions"*	1-5

 Further documents are listed in the continuation of box C. Patent family members are listed in annex

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## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>RENSHAW P.F., ET AL.: "Short-term treatment with citicoline (CDP-choline) attenuates some measures of craving in cocaine-dependent subjects: a preliminary report"  <i>PSYCHOPHARMACOLOGY</i>,  vol. 142, no. 2, February 1999 (1999-02),  pages 132-138, XP002191579  *cf. abstract, table 1 on page 133,  right-sided col. page 134, right col.,  table 2, page 137, left col., lines 23-37,  and right col., 2nd para.*</p> <p>---</p>	1-5
Y	<p>PATT, S, CERVOS-NAVARRO J., ET AL.: "The effects of CDP-choline on newborn rat pups with experimental alcohol fetopathy. A Golgi study"  <i>HISTOLOGY AND HISTOPATHOLOGY</i>,  vol. 4, no. 4, October 1989 (1989-10),  pages 429-434, XP002191580  *cf. summary, page 433, right-sided col.,  4th para. bridging with page 434, left  col.*</p> <p>-----</p>	1-5

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## Information on patent family members

International Application No.

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9907385	A 18-02-1999	US 5958896 A	28-09-1999
		AU 8768398 A	01-03-1999
		BR 9811876 A	02-01-2002
		EP 1005349 A1	07-06-2000
		WO 9907385 A1	18-02-1999
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nancy lead to fetal injury and death, continued treatment with anticonvulsants is generally advisable (see Ch. 249).

**Surgical therapy.** About 10 to 20% of patients have seizures that are refractory to medical treatment. Most patients whose seizures originate from a local area of abnormal brain function improve markedly when the epileptic focus is resected. Some are completely cured. Because extensive monitoring and skilled medical-surgical teamwork are required, these patients are best managed in specialized centers.

**Vagus nerve stimulation:** Intermittent electrical stimulation of the left vagus nerve with an implanted pacemaker-like device reduces the number of partial seizures by one third. After the device is programmed, patients can activate it with a magnet when they sense a seizure is imminent. Vagus nerve stimulation is used as an adjunct to an anticonvulsant. Adverse effects include a deepening of the voice during stimulation, cough, and hoarseness. Complications are minimal. Duration of effectiveness is not well established.

## 173 / SLEEP DISORDERS

*Disturbances that affect the ability to fall or stay asleep, that involve sleeping too much, or that result in abnormal sleep-related behavior.*

*(See also Sleep Problems and Nocturnal Enuresis under BEHAVIORAL PROBLEMS in Ch. 262.)*

Although sleep is necessary for survival, its precise homeostatic contribution is unknown. Individual requirements vary widely, ranging from 4 to 10 h every 24 h in healthy persons. Several factors, including current emotional state and age, influence the duration and satisfaction of sleep.

The two types of sleep, nonrapid eye movement (NREM) and rapid eye movement (REM) sleep, are marked by characteristic EEG and other changes, including eye movements. NREM sleep (75 to 80% of total sleep

time) normally initiates sleep, is characterized by slow waves (in stages 2 to 4) on an EEG, and ranges in depth from stage 1 to 4 (the deepest level), with commensurate difficulty in arousal. Muscle tone, BP, and heart and respiratory rates are reduced. REM sleep produces low-voltage fast activity on an EEG. Rate and depth of respiration fluctuate, and muscle tone is further reduced. During a normal night's sleep, REM sleep follows each of 4 to 6 cycles of NREM sleep (see Fig. 173-1). Most dreaming occurs dur-

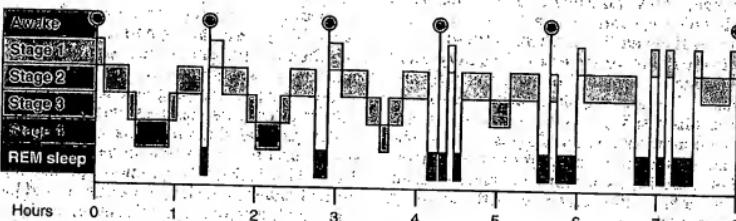


FIG. 173-1. Sleep stages in an adult during a single night. Rapid eye movement (REM) sleep occurs cyclically throughout the night every 90 to 120 min. Stage 1 accounts for 2 to 5% of the time; stage 2, for 45 to 55%; stage 3, for 3 to 8%; stage 4, for 10 to 15%; and REM, for 20 to 25%. Brief awakenings normally occur throughout the night, especially at the end of each sleep cycle.

ing REM sleep; most night terrors, sleep-walking, and talking occur during stages 3 and 4 NREM sleep.

## INSOMNIA

*Difficulty in falling asleep or in staying asleep or disturbed sleep patterns resulting in insufficient sleep.*

With advancing age, total sleep time tends to decrease. Stage 4 may disappear; sleep becomes more interrupted. These changes, although normal, may be distressing and lead to a request for treatment, but there is no evidence that they interfere with health.

Insomnia is a common symptom; about 10% of the population have chronic insomnia, and about 50% have significant insomnia at some time. It may be primary (ie, long-standing, with little or no apparent relationship to immediate somatic or psychic events) or secondary to emotional problems, pain, physical disorders, or use or withdrawal of drugs. Excess alcohol consumed in the evening can shorten sleep and lead to withdrawal effects in the early morning, so that the patient is restless on awakening or, if severely dependent, fearful and tremulous.

**Initial insomnia** (difficulty in falling asleep) is commonly associated with an emotional disturbance (eg, anxiety, a phobic state, depression), pain, respiratory problems, stimulant drugs, withdrawal of sedative drugs, poor sleep hygiene (eg, a variable sleep schedule), and sleep disorders (eg, restless legs syndrome, sleep apnea, delayed sleep phase syndrome). Delayed sleep phase syndrome is a circadian rhythm disturbance in which the patient has delayed sleep and waking times and cannot advance his sleep schedule (ie, cannot move to an earlier bed-time with an earlier awakening time).

In **early morning awakening**, the patient falls asleep normally but awakens early and cannot fall asleep again or drifts into a restless, unsatisfying sleep. This pattern is a common phenomenon of aging but is sometimes associated with depression. Tendencies to anxiety, self-reproach, and self-punitive thinking, often magnified in the morning, may contribute.

**Sleep rhythm reversals** usually reflect a circadian rhythm disorder (eg, jet lag—see Ch. 283) or damage to the hypothalamic region of the diencephalon (eg, after severe head injury or encephalitis). Misusing seda-

tives or working irregular night-shift hours can sometimes induce such reversals, as can obstructive sleep apnea. Patients with obstructive sleep apnea become drowsy in the morning, sleep or doze much of the day, and have fitful, interrupted sleep at night. If the dose of a sedative is increased because of an incorrect diagnosis, restlessness and wandering in a clouded or confused state may occur at night.

**Rebound wakefulness** commonly occurs when hypnotics are withdrawn from a patient who regularly takes heavy doses. Most patients wrongly interpret this effect as recurrence of chronic insomnia.

### Diagnosis

The cause (there may be several) should be determined by evaluating the patient's sleep pattern, use of drugs (including alcohol, caffeine, and nicotine), degree of psychologic stress, and level of physical activity. When insomnia is an isolated symptom, the only likely physical cause is a primary sleep apnea syndrome (see below). However, insomnia may reflect a normal need for little sleep. An irregular schedule (eg, from shift work, during travel, or on weekends) in an otherwise normal person can lead to insomnia. Difficulty falling asleep is usually due to anxiety. Early morning awakening or insomnia unresponsive to simple corrective measures is often due to a significant emotional disturbance (especially depression) or a physical disorder (eg, pain or a respiratory disturbance during sleep). For persistent unexplained insomnia, evaluation in a sleep laboratory may help.

### Treatment

Treatment depends on the underlying cause. Many patients respond to reassurance that their sleeplessness is a result of normal anxieties or is secondary to a treatable physical disorder. Discussing anxieties with the patient often eases distress and helps reestablish normal sleep patterns. Simple measures can often help relieve insomnia (see TABLE 173-1). Elderly patients experiencing normal changes in sleep patterns should be reassured, instructed in relaxation, and encouraged to exercise more during the day and to avoid daytime naps. Warm milk helps some patients sleep.

For insomnia due to emotional disturbance other than depression and for refrac-

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tory cases, a hypnotic drug may be temporarily required, especially if sleeplessness impairs the patient's efficiency and sense of well-being. The patient should be urged to use hypnotics only short term (2 to 4 wk) or episodically (no more than a few times a week), because tolerance and addiction can result. Depressed patients should be given limited amounts to reduce the risk of attempting suicide with the hypnotic. Patients who awaken because of pain should receive analgesics at bedtime as the mainstay of treatment. For insomnia accompanying depression, a sedating tricyclic antidepressant taken about 1 h before bedtime is usually best (see Ch. 189).

**Hypnotics:** Terms such as hypnotic, sedative, antianxiety drug, minor tranquilizer, and anxiolytic are often used interchangeably. The best term for drugs used primarily to induce sleep is hypnotic. All hypnotics pose some risk of overdose, habituation, tolerance, addiction, and withdrawal. For those with the greatest effectiveness and safety, see TABLE 173-2.

Adverse effects include drowsiness, lethargy, hangover, and amnesia, especially after excessive intake of certain hypnotics. After using such drugs, ambulatory patients should avoid activities requiring mental alertness, judgment, and physical coordination (eg, driving a vehicle, operating machinery) for several hours (generally about 8 to 12 h, but sometimes longer). Hypnotics should be used with caution in patients with pulmonary insufficiency. Rarely, skin eruptions (eg, urticaria, angioneurotic edema, bullous erythema multiforme) and GI disturbances (eg, nausea, vomiting) occur. In the elderly, any hypnotic, even in small doses, can cause restlessness, excitement, or exacerbations of delirium and dementia.

Many patients take higher doses of hypnotics than they admit; slurring of speech, incoordination, tremulousness, and nystagmus should arouse suspicion of overdosage. If necessary, serum levels of many drugs can be measured.

Hypnotics are additive in effect with other CNS depressants (eg, alcohol, antianxiety drugs, opioids, antihistamines, antipsychotics, antidepressants). Doses should be reduced when these drugs are used concurrently. Sudden withdrawal after prolonged use may precipitate severe tremors or seizures. Barbiturates, chloral hydrate, chloral

TABLE 173-1. WAYS TO IMPROVE SLEEP

Regular sleep schedule	Bedtime and wake-up time should be the same each day, including weekends.
Regular bedtime routine	A pattern of activities—brushing teeth, washing, setting the alarm clock—can set the mood for sleep.
Sleep-conducive environment	The bedroom should be dark, quiet, and reasonably cool; it should be used only for sleep and sexual activity, not for eating, reading, watching television, or paying bills. Heavy curtains or a sleep mask can eliminate light, and earplugs, fans, or special white-noise devices can help eliminate disturbing noise.
Pillows	Pillows between the knees or under the waist can make a person more comfortable. For persons with back problems, lying supine with a large pillow under the knees can help.
Regular exercise	Exercise promotes sleep and reduces stress, but if performed in the late evening, it can stimulate the cardiovascular and nervous systems and interfere with falling asleep.
Relaxation	Stress and worry interfere with sleep. Reading or taking a warm bath before bedtime can help a person relax. Techniques such as visual imagery, progressive muscle relaxation, and breathing exercises can be used.
Avoidance of stimulants and diuretics	Drinking alcoholic or caffeine-ated beverages, smoking, eating caffeinated foods (eg, chocolate), taking appetite suppressants, and taking prescription diuretics—especially near bedtime—should be avoided.

betaine, and glutethimide can interact with coumarin anticoagulants. For other potential problems of hypnotic drug use, see Ch. 190 and ANXIOLYTIC AND HYPNOTIC DRUG DEPENDENCE in Ch. 195.

Many benzodiazepine derivatives have a marked hypnotic effect. They have minimal

TABLE 173-2. HYPNOTICS IN COMMON USE

Drug	Half-life (h)	Advantages and Disadvantages	Dose* (mg)
<b>Benzodiazepines</b>			
Midazolam	1.5-2.3	Doses > 15 mg may lead to rebound insomnia	7.5-15
Triazolam	1.5-3	Useful for initial insomnia; high doses induce anterograde amnesia	0.125-0.25
Oxazepam	6-9.5	Slowly absorbed; useful for sleep-maintenance insomnia	10-30
Temazepam	8-9	Useful for initial insomnia; few residual effects with 10 mg	10-20
Lorazepam	10-20	Intermediate-length sedation	2-4
Estazolam	16-18	Few residual effects in dose range	0.5-2
Nitrazepam <sup>†</sup>	25-35	Useful for frequent awakenings when some daytime sedation is acceptable	2.5-10
Diazepam <sup>†</sup>	30-56	Accumulates because of slow elimination of drug and its active metabolite	2.5-10
Quazepam	39	Prolonged use not recommended; may be useful for early morning awakening	7.5-15
Flurazepam <sup>†</sup>	40-100	Useful for frequent awakenings when some daytime sedation is acceptable	15-30
Clorazepate <sup>†</sup>	55-70	Useful for insomnia with anxiety	7.5-22.5
<b>Imidazopyridine</b>			
Zolpidem	1.5	Few residual effects with doses up to 30 mg	5-10
<b>Antidepressants<sup>‡</sup></b>			
Amitriptyline	16	Full doses at bedtime may ameliorate insomnia in patients with depression and early morning awakening; strong anticholinergic properties	50-100
Doxepin	11-23	Sedating; strong anticholinergic properties	50-100
<b>Others</b>			
Chloral hydrate	4-10	Intermediate-length sedation; may have GI and residual effects	500-1000
Meprobamate	6-17	Mild sedation; useful in elderly; may have residual effects	800
<b>Nonprescription hypnotics</b>			
Diphenhydramine <sup>§</sup> , pyrilamine, etc		Mild sedation with most, but sedative effect lost after 3-4 days of use; strong anticholinergic properties (dry mouth, blurred vision, urinary retention, constipation), which are particularly problematic in the elderly and in patients with glaucoma, benign prostatic hyperplasia, or dementia	25-50

\*For elderly patients, initial doses at 1/2 the minimal dose often suffice.

<sup>†</sup>Drug should be avoided in the elderly because of age-related long half-life.<sup>‡</sup>Antidepressants should not be used unless depression is present.<sup>§</sup>Drug should be avoided in the elderly because of strong anticholinergic properties.

ose\* (mg)

5-15

125-0.25

-30

-20

4

-2

-10

-10

-15

30

22.5

0

0

0

0

0

0

0

suicidal risk when used alone and do not cause REM cycles to increase after discontinuation; high doses can decrease slow-wave sleep and alter REM sleep; and medium-sized doses can induce retrograde amnesia. Short-acting benzodiazepines (eg, triazolam) are useful for initial insomnia; intermediate-acting ones (eg, estazolam, temazepam), for sleep-maintenance insomnia. Long-acting ones (flurazepam, quazepam) may be useful for early morning awakening but are more likely to impair daytime function, especially in the elderly.

Benzodiazepines have less addictive potential than other hypnotics. Doses that induce serious respiratory and vital center depression are considerably larger for benzodiazepines than for barbiturates and most other hypnotics. A few patients report an increase in daytime anxiety after repeated use or withdrawal.

Chloral hydrate is a relatively weak but safe hypnotic. The usual oral dose is 0.5 to 1 g; if necessary, an additional 0.5 g may be given after 1 h. Chloral hydrate is available in capsules and in solutions (with a pungent, unpleasant taste). It can cause tolerance and addiction and induce hepatic drug-metabolizing enzymes.

Barbiturates are not recommended as hypnotics. They predispose to tolerance, habituation, and drug dependence and have a higher suicide risk than other hypnotics (see ANXIOLYTIC AND HYPNOTIC DRUG DEPENDENCE in Ch. 195). They strongly induce hepatic drug-metabolizing enzymes. Attempts to discontinue barbiturates may lead to withdrawal symptoms, intensifying dependence on the drug. Substituting a benzodiazepine should be attempted, with gradual weaning from barbiturates.

Glutethimide and methyprylon have a fairly long duration of action and can produce tolerance and addiction. They are seldom used and not recommended. *Overdose with glutethimide is particularly likely because the toxic dose is not much larger than the hypnotic dose.*

Antihistamines are commonly used as hypnotics and are the active ingredient in almost all OTC hypnotics. However, they are strongly anticholinergic, causing constipation, urinary retention, dry mouth, orthostatic hypotension, blurred vision, and confusion, especially in the elderly. Sedating antidepressants (eg, some tri-

cyclics) should not be used as hypnotics unless depression is present.

**Melatonin.** This hormone, which is closely linked to the circadian system, is normally released by the pineal gland at night. Daily treatment of blind persons with melatonin can entrain free running rhythms if the suprachiasmatic nucleus in the hypothalamus is intact. Because melatonin can reset rhythms, it has been tested as treatment for initial insomnia and jet lag. However, it is not recommended by most sleep experts, because morning exposure to bright light is more effective than melatonin at resetting rhythms for healthy persons; available preparations of melatonin are unregulated, so content and purity cannot be assured; and the effects of long-term exposure to exogenous melatonin are unknown.

## HYPERSOMNIA

*A pathologic increase in absolute sleep hours by  $\geq 25\%$ .*

Chronic hypersomnia can result from space-occupying lesions affecting the hypothalamus or upper brain stem, increased intracranial pressure, excessive use or abuse of hypnotic drugs or some illicit drugs, or certain forms of encephalitis. It can occur as a symptom of depression. Acute, relatively brief hypersomnia commonly accompanies acute systemic diseases, such as influenza. In addition, hypothyroidism, hyperglycemia, hypoglycemia, anemia, uremia, hypercapnia, hypercalcemia, liver failure, epilepsy, and multiple sclerosis can cause hypersomnia. Patients with a sleep apnea syndrome (see below) often have compensatory daytime hypersomnia. A thorough history and examination and, when indicated, brain imaging or screening of blood and urine can identify most of these disorders. The Kleine-Levin syndrome, an extremely rare condition in adolescent boys, produces episodic hypersomnia and hyperphagia.

## NARCOLEPSY

*A rare syndrome of hypersomnia with sudden loss of muscle tone (cataplexy), sleep paralysis, and hypnagogic phenomena.*

About 10% of narcoleptics have the full tetrad of symptoms. The cause is unknown,

although all tested patients have belonged to specific HLA haplotypes, suggesting a genetic cause. Narcolepsy is equally common in both sexes; some patients have a family history of the disorder. No pathologic changes are seen in the brain. Longevity is unaffected.

### Symptoms and Signs

Symptoms usually begin in adolescents or young adults without prior illness and persist throughout life. All symptoms and signs are intensifications of normal phenomena. However, the symptoms may put the patient in danger, often interfere with work and social relationships, and can drastically reduce quality of life.

Sleep occurs anytime. Sleep episodes vary from few to many in a single day, and each episode may last minutes or hours. The patient can resist the desire to sleep only temporarily but can be roused from narcoleptic sleep as readily as from normal sleep. Sleep tends to occur during monotonous conditions conducive to normal sleep but may also occur during hazardous circumstances (eg, while driving). The patient may feel refreshed on awakening yet fall asleep again in a few minutes. Total daily sleep time usually does not increase despite the frequent sleep episodes. Onset of REM sleep is almost instantaneous. This pattern differs from normal sleep, in which NREM sleep usually lasts about 60 to 90 min, preceding REM sleep. Nighttime sleep may be unsatisfying and interrupted by vivid, frightening dreams.

Cataplexy is momentary paralysis without loss of consciousness evoked by sudden emotional reactions, such as mirth, anger, fear, or joy, or, often, by surprise. Weakness may be confined to the limbs (eg, the patient may drop the rod when a fish strikes) or may cause a limp fall when the patient laughs heartily or is suddenly angry. These attacks resemble the loss of muscle tone that occurs during REM sleep or, to a lesser degree, in a person who is "weak with laughter."

In **sleep paralysis**, the patient tries to move when just falling asleep or immediately on awakening and finds that for a moment he cannot. These occasional episodes may be very frightening. They resemble the motor inhibition that accompanies REM sleep and are common in normal children and in some otherwise normal adults.

**Hypnagogic phenomena** are particularly vivid auditory or visual illusions or hallucinations that may occur at the onset of sleep or, less often, on awakening. They are difficult to distinguish from intense reverie and are somewhat similar to vivid dreams, which are normal in REM sleep. Hypnagogic phenomena occur commonly in young children and occasionally in adults who do not have narcolepsy or other sleep disorders.

The only specific laboratory abnormality is low-voltage fast activity (typical of REM sleep) during an episode.

### Diagnosis

A history of typical episodes is characteristic, and other symptoms of the clinical tetrad should be sought. With the history, a multiple sleep latency test can usually confirm the diagnosis. A few patients have sleep episodes only and lack the typical early onset of REM sleep.

Specific causes of hypersomnia must be ruled out. Sleep deprivation and depression are differentiated by evaluating the psychiatric history, circumstances of sleep, and duration of symptoms and by noting lack of cataplexy.

### Treatment

Many otherwise normal persons have occasional episodes of sleep paralysis or hypnagogic phenomena, are not bothered, do not seek medical assistance, and need no treatment. For other persons, modafinil or stimulant drugs may help prevent sleepiness. Modafinil is given as a single morning dose of 200 or 400 mg po. Dosage of stimulants is based on individual need. Methylphenidate 20 to 60 mg/day po in divided doses during the day may be most effective; ephedrine 25 mg, amphetamine 10 to 20 mg, or dextroamphetamine 5 to 10 mg po q3 to 4 h may be used. Tricyclic antidepressants (particularly imipramine, clomipramine, and protriptyline) and monoamine oxidase inhibitors are useful in treating cataplexy, sleep paralysis, and hypnagogic hallucinations. Imipramine 10 to 75 mg/day po is the drug of choice to treat cataplexy but should be taken only during the day to reduce nocturnal arousal. Patients taking both imipramine and stimulants risk developing hypertension and should be closely monitored.

F. “Normalize” (Merriam-Webster Dictionary) – entered June 20, 2005

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Main Entry: **nor·mal·ize**

Pronunciation: *nor-məlīz'*

Function: *transitive verb*

Inflected Form(s): **nor·mal·ized**; **nor·mal·iz·ing**

Date: 1865

1 : to make conform to or reduce to a norm or standard  
2 : to make **normal** (as by a transformation of variables)  
3 : to bring or restore (as relations between countries) to a **normal** condition  
— **nor·mal·iz·a·ble**   
— **nor·mal·iza·tion**

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G. "Physical condition" (PubMed search) – entered November 3, 2006



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H. Radulovacki et al. (*J. Pharmacol. Exper. Ther.* 228:268-274 (1984)) – entered  
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# Adenosine Analogs and Sleep in Rats<sup>1</sup>

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Accepted for publication October 19, 1983

## ABSTRACT

The effects of  $N^6$ -L-(phenylisopropyl)adenosine, cyclohexyladenosine and adenosine-5'-N-ethylcarboxamide on sleep were examined in rats. These effects consist of 1) increased slow-wave sleep; from 8.8 to 45.7% in all doses used for cyclohexyladenosine and adenosine-5'-N-ethylcarboxamide and for 0.1 and 0.5  $\mu$ mol/kg of  $N^6$ -L-(phenylisopropyl)adenosine and 2) increased values for rapid-eye-movement-sleep, amounting to 56.2 and 51.6% for 0.1  $\mu$ mol/kg of cyclohexyladenosine and 0.3  $\mu$ mol/kg of  $N^6$ -L-(phenylisopropyl)adenosine, respectively. Slow-wave sleep, decreased but values for wakefulness and total sleep were unchanged for 0.03, 0.1 and 0.3- $\mu$ mol/kg doses of the drugs. Only 0.9- $\mu$ mol/kg dose of cyclohexyladenosine and  $N^6$ -L-(phenylisopropyl)adenosine increased wakefulness and decreased total sleep, whereas the same dose of adenosine-5'-N-ethylcarboxamide increased total sleep during the 0- to 3-hr time

interval. All three agents reduced rapid-eye-movement sleep at the 0.6- $\mu$ mol/kg dose. The results indicate that the effect on sleep of all three adenosine analogs was enhanced with nanomolar doses of the drugs and that it diminished or disappeared when the drug dose reached micromolar range (0.9  $\mu$ mol/kg). It appears, therefore, that the effect is mainly of  $A_1$  rather than  $A_2$  receptors contributed to the sleep effects of the drugs because adenosine and adenosine analogs activate  $A_1$  receptors in nanomolar quantities whereas activation of  $A_2$  receptors requires micromolar concentration of these compounds. The results also indicate that the effects on sleep of adenosine analogs in rats differ from the effects on sleep of barbiturates and benzodiazepines, because the latter do not increase rapid-eye-movement sleep and because increases in slow-wave sleep and rapid-eye-movement sleep by adenosine analogs were obtained only at certain dosages and could not be further augmented by a dose increase.

The role of adenosine in the CNS has attracted considerable attention since the discovery that methylxanthines competitively inhibit the effects of adenosine in the CNS (Saitta and Rall, 1970). The widely accepted view today is that excitatory action of methylxanthines is due to their antagonism at adenosine receptors (Rall, 1980) rather than to the inhibition of phosphodiesterase, because concentrations of caffeine and theophylline which produce behavioral stimulation in the CNS cause only insignificant inhibition of that enzyme in the brain (Daly, 1977). Recent findings have shown that iontophoretic application of adenosine has potent depressant effects on the responses of neurons in several brain structures (Phillip and Wu, 1981) and some experimental evidence suggests that adenosine may have a role in sleep. Preliminary data in dogs (Haufler et al., 1973) indicate a possible hypnotic role for adenosine whereas administration of adenosine into the brains of rats, cats and cows produced sleep (Buday et al., 1961; Feldberg and Sherwood, 1964; Marley and Nistico, 1972). Administration of relatively low doses of adenosine analogs to

mice and rats produced marked sedation and hypothermia (Dunwidde and Worth, 1982; Snyder et al., 1981). In accordance, administration of adenosine triphosphate to rabbits or mice caused sedation (Bhattacharya et al., 1970; Mathieu-Levy, 1968).

Our interest in the possible hypnotic role of adenosine was stimulated by findings that behavioral stimulant effects of methylxanthines involve a blockade of central adenosine receptors (Snyder et al., 1981). In addition, general neurophysiologic effects of adenosine were shown to be inhibitory (Phillip et al., 1973; Stoen, 1981) and it is conceivable that stimulation of adenosine receptors may produce sedation or sleep. As adenosine is rapidly metabolized by adenosine deaminase, we administered metabolically stable adenosine analogs, L-PIA, NECA and CHA, at rates and monitored both EEG and behavior. In a previous study we reported that administration of a low dose of L-PIA increased behaviorally deep  $S_2$ , but failed to produce the same effect in the presence of caffeine (Radulovacki et al., 1982). In the present study we administered three adenosine analogs at various doses to rats and polygraphically recorded their effects on wakefulness,  $S_1$ ,  $S_2$  and REM. In addition,  $S_2$  and REM latencies, TS and  $S_2$ :TS ratio were also examined.

## Materials and Methods

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**ABBREVIATIONS:** CNS, central nervous system; L-PIA,  $N^6$ -L-(phenylisopropyl)-adenosine; NECA, adenosine-5'-N-ethylcarboxamide; CHA, cyclohexyladenosine; EEG, electroencephalogram; SWS, slow-wave sleep,  $S_1$ , SWS 2;  $S_2$ , SWS 1; REM, rapid eye movement sleep; TS, total sleep; EMG, electromyogram.

7/24

## Materials and Methods

**Animals.** Subjects were 200 to 350 g adult male Sprague-Dawley rats obtained from King Animal Laboratories, Inc. (Oregon, WI). Upon receipt, the rats were housed in cages with food and water provided ad libitum, and kept in a temperature controlled room under a 12 hr light-dark cycle, with the light period from 0600 A.M. to 0800 P.M.

Implantation of electrodes and polygraph recording. Electrodes for polygraphic recording of the EEG and EMG were implanted in 25 rats. The implantation procedure was performed under sodium pentobarbital anesthesia (40 mg/kg i.p.) which was supplemented with diethyl ether. Atropine muscarinic (2 mg/kg i.v.) was given before anesthesia to prevent respiratory distress. The electrodes for EEG recording consisted of a pair of stainless-steel screws (size 0.20  $\times$  1/4 inch) with heads soldered to them. These electrodes were threaded into the skull above the parietal cortex for subsequent bipolar EEG recording. Two additional screws were threaded, one into the occipital bone and the other into the frontal bone, to anchor the implant. For bone and two ball electrodes made from multistranded stainless-steel wire (size AWG-26) were inserted bilaterally into the dorsal neck muscles. Leads from these electrodes were then soldered to a miniature connector (Minifittech, Inc., Bloomsburg, PA) which was fixed to the skull with dental cement. Before closing of the incision, an antibiotic ointment was applied to prevent scalp infections.

After surgery, animals were housed in individual cages. Recordings were carried out at least 1 week after electrode implantation. Two days before the sleep evaluation, all rats were habituated to a cage system similar to that used in the actual recording. In addition, animals were handled at this time to minimize the stress involved in subsequent experimental procedures, i.e., drug injection and cable attachment.

On the day of the experiments, rats were placed under a beam equipped with a series of mercury slip-slings from which cable connectors were suspended into their cages. The cages were not covered but the walls of the cage were extended by adding a bottomless cage on top of the cage in which the rat was housed. In this situation each animal could be recorded in its own cage with relatively free movement. Fifteen minutes before the start of the recordings, rats received i.p. injections of either control or drug solutions. All recordings began at 10:00 A.M. and lasted for 6 hr which is a sufficient period to determine normal sleep cycles as well as a possible hypnotic effect. A closed-circuit television camera permitted the observation of animals during the study.

Recordings were made at a chart speed of 6 mm/sec, using a Grass Polygraph model 78D located outside the recording room. The half-amplitude frequency response was 1 to 60 Hz for the EEG and 30 to 80 Hz for the EMG. The sensitivity of the amplifier was adjusted for each animal at the beginning of the experiment in order to obtain the clearest distinction between sleep-wake states.

Assessment of polygraphic recordings. The effects of drug administration on the sleep-wake cycle were evaluated by assigning the predominant sleep-wake activity of each 30-sec epoch of record to either wakefulness,  $S_1$ ,  $S_2$  or REM. Wakefulness is characterized by a high frequency, low amplitude EEG and relatively high EMG tone.  $S_1$  is characterized by episodic and less than 50% high amplitude, low frequency EEG delta slow waves, whereas  $S_2$  is defined as having 50% or more delta slow waves. REM is characterized by a high frequency, low amplitude EEG and, except for occasional muscle twitches, an absence of EMG tone.  $S_2$ -latency is undefined as the time from the injection of saline or drug to the appearance of the first  $S_2$ -min for sleep episodes and REM latency was defined as the time between the injection and the appearance of the first full 1-min episode of REM.

Drugs and treatment regimen. The drugs employed in these experiments were L-PIA (Boehringer-Mannheim Corp., Indianapolis, IN), CHA (Calbiochem-Behring, La Jolla, CA), and NECA (generous gift of Dr. H. Stein, Abbott Laboratories, North Chicago, IL). All drugs were dissolved in 0.9% saline and were injected i.p. in a volume of 1.0 ml/kg (with concentrations dependent on the various doses) at 2:45 A.M. In all cases, animals in the control group received equimolar i.p. injections of 0.9% saline at the same time (2:45 A.M.) as the drug

injections. All drug concentrations are expressed in terms of the free base.

Statistical analyses. All data were analyzed using analysis of variance techniques appropriate to the experimental design. Multiple comparisons between means were made using the Student-Newman-Keuls procedure. All statistical analyses were performed according to methods previously described (Bauduer and Coohran, 1967; Winsor, 1971).

## Results

### Dose Response Effects on L-PIA

Effects on wakefulness. As shown in table 1, the 0.1 and 0.3- $\mu$ mol/kg doses of L-PIA had no statistically significant effects on wakefulness. Wakefulness was significantly increased during both the 2- to 5- and 0- to 6-hr periods by the 0.9- $\mu$ mol/kg dose of L-PIA as compared with the saline groups (+28.1 and +38.6%, respectively,  $P < .05$  in each case). This same dose also increased wakefulness during the 0- to 3- and 0- to 6-hr recording periods as compared with the 0.3- $\mu$ mol/kg dose of L-PIA (+46.0 and +38.5%, respectively,  $P < .01$  in both cases) and during the entire 6 hr of recording as compared with the 0.1- $\mu$ mol/kg dose (+32.3%,  $P < .05$ ).

Effects on  $S_1$ . All doses of L-PIA employed significantly decreased the amount of  $S_1$  during all time periods examined. The magnitude of this observed suppression of  $S_1$  ranged from a minimum of 28.1% with the 0.3- $\mu$ mol/kg dose during the 0- to 6-hr period to a maximum of 39.3% with the 0.3- $\mu$ mol/kg dose during the 0- to 6-hr period ( $P < .01$  in each case).

Effects on  $S_2$ .  $S_2$  was significantly increased during all time intervals after the administration of 0.5- $\mu$ mol/kg i.p. of L-PIA. The smallest increase in  $S_2$  was produced during the 3- to 6-hr period (+29.2%) and the largest occurred during the 0- to 3-hr recording period (+30.8%) ( $P < .01$  in all cases).  $S_2$  was significantly reduced by the 0.9- $\mu$ mol/kg dose of L-PIA as compared with the 0.3- $\mu$ mol/kg dose during both the 0- to 6-hr and 0- to 6-hr periods (-4.2 and -10.2%, respectively,  $P < .05$  in both cases).

Effects on REM. REM was increased during both the 3- to

TABLE 1  
Dose-response effects of L-PIA on wakefulness (W),  $S_1$ ,  $S_2$ , REM and TS

Behavior	W	Saline	L-PIA Dose ( $\mu$ mol/kg)	
			0.1	0.3
W	0-3	44.2 $\pm$ 10.2	44.6 $\pm$ 4.6	39.9 $\pm$ 4.0
	3-6	51.7 $\pm$ 5.1	52.0 $\pm$ 5.1	52.8 $\pm$ 4.6
	0-6	78.0 $\pm$ 10.0	73.3 $\pm$ 5.2	53.2 $\pm$ 5.8
$S_1$	0-3	26.2 $\pm$ 4.2	20.6 $\pm$ 2.9	34.7 $\pm$ 5.5 <sup>a</sup>
	3-6	26.2 $\pm$ 4.2	27.0 $\pm$ 2.3	30.7 $\pm$ 5.2 <sup>a</sup>
	0-6	107.5 $\pm$ 8.1	66.8 $\pm$ 5.1 <sup>a</sup>	65.4 $\pm$ 5.5 <sup>a</sup>
$S_2$	0-3	73.5 $\pm$ 7.8	91.0 $\pm$ 5.2	95.9 $\pm$ 4.0 <sup>a</sup>
	3-6	76.7 $\pm$ 8.0	81.0 $\pm$ 3.7	89.5 $\pm$ 5.1 <sup>a</sup>
	0-6	150.3 $\pm$ 10.7	176.0 $\pm$ 10.7	150.0 $\pm$ 10.7
REM	0-3	11.7 $\pm$ 1.8	12.7 $\pm$ 1.6	12.0 $\pm$ 0.7 <sup>a</sup>
	3-6	14.4 $\pm$ 2.1	13.7 $\pm$ 1.7	12.7 $\pm$ 0.7 <sup>a</sup>
	0-6	26.6 $\pm$ 3.7	34.4 $\pm$ 3.0	40.3 $\pm$ 4.0 <sup>a</sup>
TS	0-3	195.7 $\pm$ 10.3	125.4 $\pm$ 4.6	143.7 $\pm$ 4.0 <sup>a</sup>
	3-6	143.2 $\pm$ 6.1	145.3 $\pm$ 6.0	154.0 $\pm$ 2.9 <sup>a</sup>
	0-6	284.0 $\pm$ 12.0	280.7 $\pm$ 8.2	259.7 $\pm$ 3.5 <sup>a</sup>

Significantly different from saline control group, <sup>a</sup> $P < .05$ ; <sup>b</sup> $P < .01$ .

Significantly different from 0.1- $\mu$ mol/kg L-PIA group, <sup>a</sup> $P < .05$ ; <sup>b</sup> $P < .01$ .

Significantly different from 0.3- $\mu$ mol/kg L-PIA group, <sup>a</sup> $P < .05$ ; <sup>b</sup> $P < .01$ .

6 and 0- to 6-hr recording periods after the 0.3- $\mu$ mol/kg dose of L-PIA (+83.5%,  $P < .01$  and +51.8%,  $P < .05$ , respectively) when compared with the saline control group. The administration of 0.9  $\mu$ mol/kg of L-PIA significantly decreased REM during the 0- to 8-hr period when compared with all other experimental groups. This suppression of REM averaged 87.0% and was highly significant ( $P < .01$ ) in all cases. In addition, this dose of L-PIA also significantly reduced REM during both the 0- to 6- (−40.1%,  $P < .05$ ) and 0- to 6-hr (−55.9%,  $P < .01$ ) recording periods as compared with the 0.8- $\mu$ mol/kg dose and during the 0- to 8-hr period as compared with the 0.1- $\mu$ mol/kg dose (−47.7%,  $P < .01$ ).

**Effects on TS.** No statistically significant changes in TS were produced by the 0.1- and 0.3- $\mu$ mol/kg doses of L-PIA during any recording period. The 0.9- $\mu$ mol/kg dose significantly decreased TS during the 0.3-hr period as compared with both the saline (−10.0%) and 0.6- $\mu$ mol/kg (−19.2%) dose groups ( $P < .01$  in both cases). TS also was decreased during the 0- to 6-hr period by 0.9  $\mu$ mol/kg of L-PIA as compared with all other treatment groups and these decreases ranged from 9.3% at the 0.1- $\mu$ mol/kg dose ( $P < .05$ ) to a maximum of 14.4% at the 0.3- $\mu$ mol/kg dose ( $P < .01$ ).

**Effects on sleep latencies.** As shown in table 4, neither the 0.1- nor the 0.3- $\mu$ mol/kg dose of L-PIA produced any significant changes in sleep latencies. However, the 0.9- $\mu$ mol/kg dose of this drug significantly delayed the onset of both S<sub>1</sub> and REM as compared with all other experimental groups. These increases in sleep latencies ranged from 72.7% as compared with saline, to 169.4% as compared with the 0.1- $\mu$ mol/kg dose of L-PIA for S<sub>1</sub> latency ( $P < .01$  in both cases). REM latency was increased by a minimum of 204.3% as compared with the 0.1- $\mu$ mol/kg dose and by a maximum of 261.2% as compared with the 0.3- $\mu$ mol/kg dose group ( $P < .01$  in each case).

**Effects on ratios of minutes of S<sub>2</sub> to minutes of S<sub>1</sub>.** The dose-response effects of L-PIA on the ratios of minutes of S<sub>2</sub> to minutes of S<sub>1</sub> are summarized in table 5. The 0.1- $\mu$ mol/kg dose of L-PIA significantly increased the ratio of S<sub>2</sub>:S<sub>1</sub> during both the 0- to 3- and 0- to 6-hr period of recording (+133.3 and +86.7%, respectively,  $P < .01$  in each case). Administration of 0.3  $\mu$ mol/kg of L-PIA significantly increased S<sub>2</sub>:S<sub>1</sub> ratios during all time periods, ranging from +100% ( $P < .05$ ) during the first 3 hr to +113.3% ( $P < .01$ ) during the 0- to 6-hr recording period, as compared with the saline control group. The 0.9- $\mu$ mol/kg dose of L-PIA also significantly increased the ratios of S<sub>2</sub>:S<sub>1</sub> as compared with the control groups during both the 3- to 6- and 0- to 6-hr recording periods (+92.5 and +68.7%, respectively,  $P < .05$  in both cases). In contrast, this highest dose of L-PIA significantly reduced the S<sub>2</sub>:S<sub>1</sub> ratios during both the 0- to 3- and 0- to 6-hr periods as compared with both 0.1 and (−37.1 and −26.7%, respectively) and 0.3- $\mu$ mol/kg (−10.1 and −21.9%, respectively) doses of L-PIA ( $P < .05$  in all cases).

#### Dose-Response Effects of CHA

**Effects on wakefulness.** Table 2 shows the dose-response effects of CHA on wakefulness and the various stages of sleep. CHA did not affect wakefulness when administered at the 0.05-, 0.1-, and 0.3- $\mu$ mol/kg doses. The highest dose of CHA (0.5  $\mu$ mol/kg) significantly increased wakefulness during the 0- to 8-hr recording period as compared with both the saline control (+100.5%,  $P < .01$ ) and 0.1- $\mu$ mol/kg dose (+116.3%,  $P < .05$ ) groups. This 0.5- $\mu$ mol/kg dose also significantly in-

creased the amount of wakefulness when compared with all other experimental groups ( $P < .01$  in every case) during both the 3- to 6- and 0- to 6-hr recording periods.

**Effects on S<sub>1</sub>.** The i.p. administration of 0.03  $\mu$ mol/kg of CHA produced a highly significant suppression of S<sub>1</sub>, relative to the saline control groups, during all time periods examined ( $P < .01$  in every case) which was maximal during the 3- to 6-hr period (−61.3%). The 0.1- $\mu$ mol/kg dose of CHA significantly reduced S<sub>1</sub> as comparison with the saline control group only during the 0- to 3- (−32.3%,  $P < .01$ ) and 0- to 6-hr (−32.8%,  $P < .05$ ) recording periods. S<sub>1</sub> also was significantly suppressed during all time periods by the 0.3- $\mu$ mol/kg dose of CHA; the greatest decrease occurred during the second 3 hr of recording (−38.0%,  $P < .05$ ) with the smallest increase during the first 3 hr (−38.4%,  $P < .01$ ). Similarly, the 0.3- $\mu$ mol/kg CHA dose suppressed S<sub>1</sub> during all time periods ( $P < .01$  in all cases), again with maximum suppression during the 0- to 2-hr period (−49.2%) and minimum suppression during the 3- to 6-hr period 0.8- $\mu$ mol/kg doses of CHA also produced significantly more S<sub>1</sub> than did the saline control group during both the 3- to 6- (+28.5 and +28.4%, respectively) and 0- to 6-hr (+28.5 and 21.9%, respectively) recording periods ( $P < .05$  in every case). After treatment with 0.5  $\mu$ mol/kg of CHA S<sub>1</sub> was increased, as compared with the saline group, only during the second 3 hr of recording (+31.2%,  $P < .05$ ).

Significantly less S<sub>1</sub> was produced by the 0.3- $\mu$ mol/kg dose of CHA than by 0.03  $\mu$ mol/kg during both the 3- to 6- (−11.7%,  $P < .01$ ) and 0- to 6-hr (−16.3%,  $P < .05$ ) recording periods. S<sub>2</sub> also was suppressed by 0.5  $\mu$ mol/kg of CHA during the 0- to 3- (−38.4%) and 0- to 6-hr periods (−26.8%,  $P < .01$  in both cases) as compared with the 0.03  $\mu$ mol/kg of CHA treatment group. During the first 3 hr of recording the 0.3  $\mu$ mol/kg produced significantly less S<sub>2</sub> than did the administration of 0.1  $\mu$ mol/kg of CHA (−33.3%,  $P < .05$ ).

**Effects on REM.** As shown in table 2, the 0.03- and 0.8- $\mu$ mol/kg doses of CHA produced no significant changes in REM during any of the time periods examined in comparison with the saline control group. The i.p. administration of 0.1  $\mu$ mol/kg of CHA resulted in significantly more REM than did the saline control group during both the 3- to 6- (+28.1%,  $P < .01$ ) and 0- to 6-hr (+56.2%,  $P < .05$ ) recording periods. In contrast, the 0.3- $\mu$ mol/kg dose of CHA significantly suppressed REM during all recording periods in comparison with all other treatment groups ( $P < .01$  in all cases). REM was completely (i.e., −100.0%) absent during the 0- to 3-hr period of recording after the administration of 0.9  $\mu$ mol/kg of CHA and also was greatly reduced during both the 3- to 6- and 0- to 6-hr time periods with maximum reductions of −89.4 and −99.0%, respectively, in comparison with the 0.1- $\mu$ mol/kg CHA dose.

**Effects on TS.** The 8 lowest doses of CHA (0.03, 0.1 and 0.3  $\mu$ mol/kg) produced no statistically significant effects on the amount of TS during any of the recording periods examined. The administration of 0.9  $\mu$ mol/kg of CHA, however, produced significant reductions of TS during all time periods as compared with all other treatment groups ( $P < .01$  in every case). The maximum decreases in TS were observed in comparison to the 0.1- $\mu$ mol/kg dose and were −80.4% during the first 3 hr, −21.9% during the second 3 hr and −26.0% during the 0- to 6-hr recording period.

**Effects on sleep latencies.** The dose-response effects of CHA on latencies to S<sub>1</sub> and REM are summarized in table 4. As shown in table 4, both the 0.03- and 0.1- $\mu$ mol/kg doses of

TABLE 2

Dose-response effects of CHA on wakefulness (W), S<sub>1</sub>, S<sub>2</sub>, REM and TSAll values are means  $\pm$  S.E.M. (minutes) of five rats per group. All comparisons between means were made using analysis of variance and multiple comparison tests appropriate to the completely randomized design.

Behavioral State	W	CHA Dose ( $\mu$ mol/kg)			
		0.05	0.1	0.5	0.9
S <sub>1</sub>	0-3	38.6 $\pm$ 12.4	37.3 $\pm$ 4.9	35.8 $\pm$ 5.7	54.0 $\pm$ 10.2
	3-6	34.6 $\pm$ 7.9	34.6 $\pm$ 3.2	20.2 $\pm$ 8.0	23.4 $\pm$ 8.6
	6-9	73.2 $\pm$ 14.9	61.6 $\pm$ 5.3	56.0 $\pm$ 9.9	77.4 $\pm$ 13.0
S <sub>2</sub>	0-2	63.8 $\pm$ 6.5	27.2 $\pm$ 2.6 <sup>a</sup>	38.4 $\pm$ 2.9 <sup>b,c</sup>	34.2 $\pm$ 2.4 <sup>b,c</sup>
	3-6	0.10 $\pm$ 5.1	26.8 $\pm$ 6.8 <sup>a</sup>	41.0 $\pm$ 4.0 <sup>a</sup>	37.4 $\pm$ 4.0 <sup>a</sup>
	6-9	114.8 $\pm$ 10.7	54.0 $\pm$ 4.9 <sup>a</sup>	77.4 $\pm$ 5.6	78.0 $\pm$ 7.0 <sup>a</sup>
REM	0-3	78.2 $\pm$ 10.5	103.0 $\pm$ 6.5 <sup>a</sup>	95.0 $\pm$ 8.2	81.2 $\pm$ 9.0 <sup>a</sup>
	3-6	70.8 $\pm$ 10.1	111.2 $\pm$ 7.6 <sup>a</sup>	91.0 $\pm$ 10.0 <sup>a</sup>	86.0 $\pm$ 4.4 <sup>a,b</sup>
	6-9	147.0 $\pm$ 13.7	214.2 $\pm$ 11.0 <sup>a</sup>	166.0 $\pm$ 5.1 <sup>a</sup>	173.2 $\pm$ 10.8 <sup>a</sup>
TS	0-3	12.0 $\pm$ 2.6	12.8 $\pm$ 2.7	16.0 $\pm$ 2.1	10.6 $\pm$ 2.1
	3-6	15.0 $\pm$ 4.0	17.4 $\pm$ 2.8	25.0 $\pm$ 2.0 <sup>a</sup>	20.8 $\pm$ 3.4
	6-9	25.8 $\pm$ 5.0	30.0 $\pm$ 5.2	40.0 $\pm$ 6.4 <sup>a</sup>	51.4 $\pm$ 3.9

Significantly different from saline control group, <sup>a</sup>\*P < .05, <sup>b</sup>\*P < .01.Significantly different from 0.05- $\mu$ mol/kg CHA group, <sup>a</sup>\*P < .05, <sup>b</sup>\*P < .01.Significantly different from 0.1- $\mu$ mol/kg CHA group, <sup>a</sup>\*P < .05.Significantly different from all other treatment groups, <sup>a</sup>\*P < .01.

CHA did not significantly change the latencies to S<sub>1</sub> and REM during any recording period. The 0.6- $\mu$ mol/kg dose of CHA significantly delayed the onset of S<sub>2</sub> as compared with both with both the saline ( $+83.5\%$ , P < .05) and 0.05- $\mu$ mol/kg ( $+29.8\%$ , P < .01) treatment groups. Treatment with 0.9  $\mu$ mol/kg of CHA again significantly increased S<sub>2</sub> latencies in comparison with the saline ( $+150.5$ , P < .05) and 0.03- $\mu$ mol/kg CHA ( $+44.1\%$ , P < .01) dose groups. The administration of this highest dose of CHA also significantly increased the latency of REM when compared with all other treatment groups ( $P < .01$  in all cases), the delay in the onset of REM was greatest in comparison with the saline control group ( $+713.5\%$ ) and was least as compared with the 0.3- $\mu$ mol/kg CHA dose group ( $+232.5\%$ ).

Effects on ratios of minutes of S<sub>1</sub> to minutes of S<sub>2</sub>. As shown in table 5, the 0.03- $\mu$ mol/kg dose of CHA significantly increased the ratios of S<sub>1</sub> to S<sub>2</sub> during all time periods examined ( $P < .01$ ) and the maximum increase occurred during the second 3 hr of recording. The ratios of S<sub>1</sub> to S<sub>2</sub> were significantly reduced by 0.1  $\mu$ mol/kg of CHA as compared with the 0.03- $\mu$ mol/kg dose of CHA dose during both the 3- to 6- ( $-48.9\%$ , P < .05) and 0- to 6-hr ( $-40.6\%$ , P < .01) recording periods. The 0.3- $\mu$ mol/kg dose of CHA both significantly increased the ratio of S<sub>1</sub> to S<sub>2</sub> with respect to the saline control group ( $+83.7\%$ , P < .05) and reduced this ratio in comparison with the 0.03- $\mu$ mol/kg dose group ( $-88.1\%$ , P < .01) during the 0- to 6-hr recording period. The administration of 0.9  $\mu$ mol/kg of CHA lowered the ratios of S<sub>1</sub> to S<sub>2</sub> in comparison with the 0.03- $\mu$ mol/kg dose of CHA during both the 0- to 3- and 0- to 6-hr periods ( $-60.0\%$  and  $-42.9\%$ , respectively, P < .01 in both cases). In addition, this same CHA dose (0.9  $\mu$ mol/kg) significantly increased the ratio of S<sub>1</sub> to S<sub>2</sub> with respect to the saline control group during the 3- to 6-hr recording period ( $+157.1\%$ , P < .05).

## Dose-Response Effects of NECA

Effects on wakefulness. Table 3 summarizes the dose-response effects of NECA on wakefulness, S<sub>1</sub>, S<sub>2</sub>, REM and TS. The 0.1- and 0.3- $\mu$ mol/kg doses of NECA did not signifi-

TABLE 3  
Dose-response effects of NECA on wakefulness (W), S<sub>1</sub>, S<sub>2</sub>, REM and TSAll values are means  $\pm$  S.E.M. (minutes) of six rats per group. All comparisons between means were made using analysis of variance and multiple comparison tests appropriate to the completely randomized design.

Behavioral State	W	NECA Dose ( $\mu$ mol/kg)			
		0.1	0.3	0.9	
S <sub>1</sub>	0-3	35.5 $\pm$ 10.1	48.2 $\pm$ 4.5	33.4 $\pm$ 6.2	27.4 $\pm$ 4.3 <sup>a</sup>
	3-6	31.5 $\pm$ 7.8	27.7 $\pm$ 4.8	24.8 $\pm$ 7.6	43.9 $\pm$ 5.5 <sup>a</sup>
	6-9	70.3 $\pm$ 12.3	73.9 $\pm$ 5.4	58.5 $\pm$ 11.8	65.5 $\pm$ 6.3
S <sub>2</sub>	0-3	55.2 $\pm$ 6.5	45.3 $\pm$ 7.5	55.2 $\pm$ 7.5	68.4 $\pm$ 8.5 <sup>a</sup>
	3-6	57.7 $\pm$ 5.1	45.5 $\pm$ 5.1	27.1 $\pm$ 6.0 <sup>a,b</sup>	57.4 $\pm$ 6.0 <sup>a,b</sup>
	6-9	110.0 $\pm$ 12.5	65.1 $\pm$ 15.7	36.0 $\pm$ 9.0 <sup>a,b</sup>	68.2 $\pm$ 4.5 <sup>a</sup>
REM	0-3	78.6 $\pm$ 9.5	91.2 $\pm$ 9.5	102.0 $\pm$ 9.3	102.9 $\pm$ 4.8 <sup>a</sup>
	3-6	75.4 $\pm$ 9.5	65.2 $\pm$ 5.8 <sup>a</sup>	105.6 $\pm$ 6.8 <sup>a</sup>	94.3 $\pm$ 4.1
	6-9	162.3 $\pm$ 12.4	167.1 $\pm$ 4.8	211.6 $\pm$ 11.2	202.6 $\pm$ 6.5 <sup>a</sup>
TS	0-3	12.0 $\pm$ 2.2	5.5 $\pm$ 2.4	12.0 $\pm$ 5.5	2.5 $\pm$ 1.7
	3-6	15.0 $\pm$ 3.8	7.7 $\pm$ 3.5	24.2 $\pm$ 6.0 <sup>a</sup>	24.2 $\pm$ 5.0 <sup>a</sup>
	6-9	27.0 $\pm$ 4.4	13.0 $\pm$ 8.0	30.5 $\pm$ 3.2	16.7 $\pm$ 5.0 <sup>a</sup>

Significantly different from saline control group, <sup>a</sup>\*P < .05, <sup>b</sup>\*P < .01.Significantly different from 0.1- $\mu$ mol/kg NECA group, <sup>a</sup>\*P < .05, <sup>b</sup>\*P < .01.Significantly different from 0.3- $\mu$ mol/kg NECA group, <sup>a</sup>\*P < .05, <sup>b</sup>\*P < .01.

cantly affect wakefulness during any recording period examined. Treatment with 0.9  $\mu$ mol/kg of NECA significantly decreased wakefulness during the 0- to 3-hr period in comparison with the 0.1- $\mu$ mol/kg dose group ( $-86.3\%$ , P < .05) but increased wakefulness during the second 3 hr as compared with the 0.3- $\mu$ mol/kg NECA dose ( $+76.6\%$ , P < .05).

Effects on S<sub>1</sub>. Administration of 0.1  $\mu$ mol/kg of NECA significantly decreased the amount of S<sub>1</sub> during the 0- to 6-hr recording period as compared with the saline group ( $-19.2\%$ , P < .05). S<sub>1</sub> was also significantly suppressed by the 0.3- $\mu$ mol/kg dose of NECA in comparison with the saline control group during all recording periods and the larger decrease occurred during the second 3 hr of recording ( $-63.3\%$ , P < .01). The 0.9-

$0.1\text{-}\mu\text{mol/kg}$  dose also reduced  $S_1$  in comparison with the  $0.1\text{-}\mu\text{mol/kg}$  dose group during both the 3- to 6- and 0- to 6-hr recording period ( $-54.2$  and  $-40.4\%$ , respectively,  $P < .01$  in both cases). The  $0.9\text{-}\mu\text{mol/kg}$  dose of NECA produced a similar suppression of  $S_1$  in comparison with saline during both 3- to 6- ( $-43.3\%$ ) and 0- to 6-hr ( $-37.6$ ) periods ( $P < .01$  in each case). This dose of NECA also significantly decreased  $S_1$  in comparison with the  $0.1\text{-}\mu\text{mol/kg}$  group during the 0- to 6-hr time period ( $-22.9\%$ ,  $P < .05$ ).

Effects on  $S_2$ . As shown in table 3, the  $0.1\text{-}\mu\text{mol/kg}$  dose of NECA significantly increased  $S_2$  during both the 3- to 6- and 0- to 6-hr time periods with respect to the saline group ( $+26.9$  and  $+23.0\%$ , respectively,  $P < .05$  in each case). The administration of  $0.9\text{-}\mu\text{mol/kg}$  of NECA produced significantly more  $S_2$  during all recording periods with the maximum increase occurring during the second 3 hr of recording ( $+44.5\%$ ,  $P < .01$ ). Similarly, the  $0.8\text{-}\mu\text{mol/kg}$  dose of NECA produced more  $S_2$  than did saline treatment during both the 0- to 3- and 0- to 6-hr recording periods ( $+41.6$  and  $+33.0\%$ , respectively,  $P < .01$  in both cases). This dose of NECA also significantly increased  $S_2$  when compared with the  $0.1\text{-}\mu\text{mol/kg}$  NECA dose ( $+8.1\%$ ,  $P < .05$ ).

Effects on REM. During the second 3 hr of recording the  $0.8\text{-}\mu\text{mol/kg}$  dose of NECA significantly increased the amount of REM as compared with the  $0.1\text{-}\mu\text{mol/kg}$  dose group ( $+218.2\%$ ,  $P < .01$ ). In addition, the administration of  $0.9\text{-}\mu\text{mol/kg}$  of NECA resulted in significantly less REM than did treatment with the  $0.8\text{-}\mu\text{mol/kg}$  dose during both the 3- to 6- ( $-69.4\%$ ,  $P < .01$ ) and 0- to 6-hr ( $-72.6\%$ ,  $P < .05$ ) recording periods.

Effects on TS. Neither the  $0.1$ - nor the  $0.3\text{-}\mu\text{mol/kg}$  NECA doses produced any significant changes in TS. However, the  $0.9\text{-}\mu\text{mol/kg}$  dose reduced TS by  $10.0\%$  as compared with the saline control group ( $-D$ ).

Effects on sleep latencies. The effects of NECA on latencies to  $S_1$  and REM are presented in table 4. The  $0.9\text{-}\mu\text{mol/kg}$  dose, when compared with the saline group, significantly decreased the latency to  $S_1$  ( $-45.9\%$ ,  $P < .05$ ) but greatly delayed the onset of REM ( $+492.3\%$ ,  $P < .01$ ).

Effects on ratios of minutes of  $S_2$  to minutes of  $S_1$ . The dose-response effects of NECA on the ratios of  $S_2$  to  $S_1$  are summarized in table 5. The administration of  $0.8\text{-}\mu\text{mol/kg}$  dose of NECA significantly increased the ratios of  $S_2$  to  $S_1$  with respect to the control group during all three time periods. This increase was greatest during the second 3 hr of recording ( $+500.0\%$ ,  $P < .01$ ). The  $0.9\text{-}\mu\text{mol/kg}$  NECA dose also produced

significantly greater  $S_2$  to  $S_1$  latencies than did the  $0.1\text{-}\mu\text{mol/kg}$  dose during the 3- to 6- ( $+204.5\%$ ,  $P < .01$ ) and 0- to 6-hr ( $+181.7\%$ ,  $P < .05$ ) time periods. After the administration of  $0.9\text{-}\mu\text{mol/kg}$  of NECA, the ratios of  $S_2$  to  $S_1$  were significantly less in comparison with the  $0.3\text{-}\mu\text{mol/kg}$  dose during both the 3- to 6- and 0- to 6-hr recording periods ( $-56.7$  and  $-42.5\%$ , respectively,  $P < .05$  in both cases).

## Discussion

The results of the present study show the effects on sleep of the certain doses of all three adenosine analogs used. These effects involve 1) increased values for  $S_2$  from  $6.6$  to  $45.7\%$  within a 6-hr EEG recording period in all doses used for CHA and NECA and for  $0.1$  and  $0.3\text{-}\mu\text{mol/kg}$  of  $L\text{-PIA}$  and 2) increased values for REM amounting to  $55.2$  and  $51.9\%$  for  $0.1\text{-}\mu\text{mol/kg}$  of CHA and  $0.8\text{-}\mu\text{mol/kg}$  of  $L\text{-PIA}$ , respectively, within the same time period. The increases in  $S_2$  and REM were probably achieved by a decrease in  $S_1$ , because  $S_2/S_1$  ratios were highest with the doses that were most effective in increasing  $S_2$ . Also, values for wakefulness and TS were unchanged for the  $0.03$ ,  $0.1$ , and  $0.3\text{-}\mu\text{mol/kg}$  doses. Only  $0.8\text{-}\mu\text{mol/kg}$  dose of  $L\text{-PIA}$  and CHA increased wakefulness and decreased TS, whereas the same dose of NECA increased TS during the 0- to 3-hr time interval. All three agents reduced REM in  $0.9\text{-}\mu\text{mol/kg}$  dose, but because the amount of REM is relatively small in relation to the total recording time (which includes TS and wakefulness), the reduction could not significantly affect either sleep states or wakefulness.

These results are in agreement with our previous report in which administration of  $L\text{-PIA}$  ( $0.8\text{-}\mu\text{mol/kg}$ ) to rats increased  $S_2$  by  $54$  min within a 6-hr recording period but failed to produce the same effect in the presence of caffeine (Radulovacki et al., 1982), a finding consistent with the hypothesis that the CNS-stimulant effect of caffeine and other methylxanthines is due to their ability to antagonize depressant effects of endogenous adenosine (Phillis and Wu, 1981). If stimulation of adenosine receptors is involved in behavioral and EEG effects of  $L\text{-PIA}$ , then raising the levels of endogenous adenosine in the CNS should produce similar behavioral and EEG results. One of the agents that would be expected to elevate the levels of adenosine in the CNS is deoxycoformycin, a potent inhibitor of adenosine deaminase (Agarwal et al., 1977). In a previous experiment we administered deoxycoformycin to rats in a dose-related manner and obtained an increase in REM and  $S_2$  (Radulovacki et al., 1983). Furthermore, i.v. infusion of adenosine to rats was

TABLE 4  
Dose-response effects of adenosine analogs on latencies to  $S_1$  and REM

All values are mean  $\pm$  SEM (number of six rats per group). All comparisons between means were made using analysis of variance and multiple comparison tests appropriate to the completely randomized experimental design.

Adenosine Analog	Sleep Stage	Saline	0.03	Adenosine Average Dose ( $\mu\text{mol/kg}$ )		
				0.1	0.3	0.8
L-PIA	$S_2$	$20.9 \pm 5.9$		$43.4 \pm 4.1$	$183 \pm 4.9$	$36.1 \pm 9.6^*$
	REM	$53.5 \pm 14.1$		$65.4 \pm 12.8$	$48.0 \pm 13.5$	$165.8 \pm 15.6^*$
CHA	$S_1$	$13.4 \pm 4.7$	$6.2 \pm 7.8$	$20.0 \pm 3.2$	$24.6 \pm 7.7^*$	$33.5 \pm 10.7^*$
	REM	$43.0 \pm 14.0$	$56.6 \pm 18.2$	$50.5 \pm 15.2$	$82.8 \pm 24.2$	$349.8 \pm 10.2^*$
NECA	$S_2$	$17.0 \pm 5.2$		$11.3 \pm 3.5$	$16.0 \pm 7.5$	$9.2 \pm 3.3^*$
	REM	$43.7 \pm 12.2$		$210.7 \pm 57.4^*$	$160.5 \pm 54.5^*$	$226.5 \pm 59.5^*$

Significantly different from all other groups. \* $P < .05$ ; \*\* $P < .01$ .

Significantly different from saline control group. \* $P < .05$ ; \*\* $P < .01$ .

Significantly different from 0.03- $\mu\text{mol/kg}$  CHA group. \* $P < .01$ .

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TABLE 6  
Dose-response effects of adenosine analogs on the ratios of minutes of  $S_1$  to minutes of  $S_2$ All values are means  $\pm$  S.E.M. of six rats per group. As comparisons between means were made using analysis of variance and multiple comparison tests appropriate to the completely randomized experimental design.

Adenosine Analog	Nr	Saline	Adenosine Analog Dose (mg/kg)		
			0.01	0.1	0.5
L-PIA	0.3	1.2 $\pm$ 0.5	3.2 $\pm$ 0.8*	3.0 $\pm$ 0.4*	2.5 $\pm$ 0.5*
	3.6	1.8 $\pm$ 0.5	2.1 $\pm$ 0.3	2.5 $\pm$ 0.3*	2.5 $\pm$ 0.3*
	6.0	1.6 $\pm$ 0.5	2.1 $\pm$ 0.3	2.5 $\pm$ 0.3*	2.5 $\pm$ 0.3*
CMA	0.3	1.2 $\pm$ 0.3	4.5 $\pm$ 0.8*	2.7 $\pm$ 0.2	3.4 $\pm$ 0.4
	3.6	1.4 $\pm$ 0.3	4.5 $\pm$ 0.7*	2.3 $\pm$ 0.3	3.2 $\pm$ 0.7*
	6.0	1.4 $\pm$ 0.2	4.8 $\pm$ 0.7*	2.5 $\pm$ 0.3	3.6 $\pm$ 0.3*
NECA	0.3	1.2 $\pm$ 0.2	2.5 $\pm$ 0.3	3.2 $\pm$ 0.2*	5.1 $\pm$ 0.4
	3.6	1.8 $\pm$ 0.4	2.1 $\pm$ 0.1	6.4 $\pm$ 1.1	2.8 $\pm$ 0.5*
	6.0	1.8 $\pm$ 0.5	2.0 $\pm$ 0.1	6.4 $\pm$ 0.5	2.8 $\pm$ 0.2*

Significantly different from saline control group, \* $P < .05$ ; \*\* $P < .01$ .Significantly different from 0.01-mg/kg dose group, \* $P < .05$ ; \*\* $P < .01$ .Significantly different from 0.05-mg/kg dose group, \* $P < .05$ ; \*\* $P < .01$ .Significantly different from 0.5-mg/kg dose group, \* $P < .05$ ; \*\* $P < .01$ .

shown to decrease wakefulness and increase both  $S_2$  and  $T_3$  (Virus et al., 1984). In accordance, administration of 9-adenosyladenosine, which can be converted to adenosine in the brain, *in vivo*, increased SWS and REM when the compound was given in the afternoon hours (Sardis et al., 1988), whereas administration of the drug to cats decreased wakefulness and  $S_1$  and increased  $S_2$  and  $T_3$  in a similar dose-response relationship to our data (Foulouji et al., 1980). Also, administration of 3-adenosyl-1-methionine to rabbits produced sedation and synchronization in the cortical EEG (de Al et al., 1978).

Biological mechanisms of the effect of adenosine and adenosine analogs on sleep are not well understood. There are two types of adenosine receptors on the external surface of cells,  $A_1$  and  $A_2$  (Van Calker et al., 1979), which differ considerably in their selectivity toward adenosine and adenosine analogs. Both adenosine and adenosine analogs used here activate  $A_1$  receptors in nanomolar quantities and reduce adenosylate cyclase activity, whereas activation of  $A_2$  receptors, linked with stimulation of cAMP cyclase, requires micromolar quantities of the compounds (Van Calker et al., 1979; London et al., 1980; Snyder et al., 1981). It is of interest to note that the effect of the adenosine analogs employed on  $S_2$  and REM was obtained with nanomolar concentrations of the drugs and that it diminished when the concentration of the drugs reached micromolar range (0.5  $\mu$ mol/kg). This suggests that activation of  $A_1$  rather than  $A_2$  receptors contributed to these drug actions. In addition, adenosine may have a modulatory effect on transmembrane  $Ca^{2+}$  fluxes (Phillips, 1977) and may suppress  $Ca^{2+}$  influx into intracellular compartments (Van Brempt et al., 1977). Recently, adenosine receptor agonists have been shown to inhibit  $K^{+}$ -evoked  $Ca^{2+}$  uptake by rat brain cortical synaptosomes (Wu et al., 1989). If such a depression of  $Ca^{2+}$  flux occurs at presynaptic sites, it may reduce the release of brain neurotransmitters (Schubert et al., 1980). Thus, the effect of L-PIA, CMA and NRCA on sleep could be due to the action of these compounds on  $A_1$  receptors and reduced release of brain monoamines, as monoamines have been implicated in the regulation of sleep (Jouvet, 1969). Accordingly, adenosine has been reported to reduce the release of acetylcholine (Ginsburg and Mirsky, 1972; Ribeiro and Walker, 1975; Lekic, 1977; Gustavson et al., 1978), norepinephrine (Hedquist and Freedman, 1976; Enzwe and Saidman, 1977; Verhaeghe et al., 1977),  $\gamma$ -amino-

butyric acid (Holmes and Stone, 1980), serotonin (Harms et al., 1979) and dopamine (Michaels et al., 1979), whereas an adenosine analog, 2-chloroadenosine, decreased the amount of released dopamine (Michaels et al., 1979).

Physiological SWS or SWS produced by barbiturates, benzodiazepines or adenosine analogs in rats is characterized by the high voltage amplitude synchronization in the EEG. For this reason, synchronization in the EEG alone could not be taken as a good parameter for distinguishing between physiological and drug-induced SWS. However, hypnotic drugs have been shown to suppress REM, whereas adenosine analogs increased REM in the present study. This suggests that adenosine analogs act through a different mechanism in producing their effect on sleep than hypnotic drugs. This may suggest further that  $S_2$  induced by adenosine analogs differs from  $S_2$  of the hypnotic agents and is, perhaps, closer to the physiological  $S_2$  for at least two reasons: 1) increases in  $S_2$  and REM by adenosine analogs were obtained only at certain dosages and could not be further augmented by a dose increase and 2) adenosine modulates the activity of neurotransmitters found to contribute to wakefulness. The results indicate a role for adenosine in the regulation of sleep and hold promise for a new class of compounds as potential agents in the treatment of sleep disorders.

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I. Renshaw (U.S. Patent No. 6,103,703) – entered December 6, 2004



US006103703A

**United States Patent** [19]

Renshaw et al.

[11] **Patent Number:** 6,103,703[45] **Date of Patent:** \*Aug. 15, 2000

[54] **CYTIDINE-CONTAINING AND CYTOSINE-CONTAINING COMPOUNDS AS TREATMENTS FOR STIMULANT EXPOSURE**

[75] **Inventors:** Perry F. Renshaw, Arlington; Scott Lukas, Belmont, both of Mass.

[73] **Assignee:** The McLean Hospital Corporation, Belmont, Mass.

[\*] **Notice:** This patent is subject to a terminal disclaimer.

[21] **Appl. No.:** 09/300,107

[22] **Filed:** Apr. 27, 1999

**Related U.S. Application Data**

[63] Continuation of application No. 08/908,997, Aug. 8, 1997, Pat. No. 5,958,896.

[51] **Int. Cl. 7** ..... A61K 31/513; A61K 31/7068

[52] **U.S. Cl.** ..... 514/49; 514/274; 514/812

[58] **Field of Search** ..... 514/49, 812, 274

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(List continued on next page)

**Primary Examiner—Phyllis G. Spivack  
Attorney, Agent, or Firm—Clark & Elbing LLP**

[57]

**ABSTRACT**

Disclosed herein is a method for reducing stimulant dependencies in mammals that involves administration of a therapeutically-effective amount of a cytosine-containing or cytidine-containing compound, such as CDP-choline.

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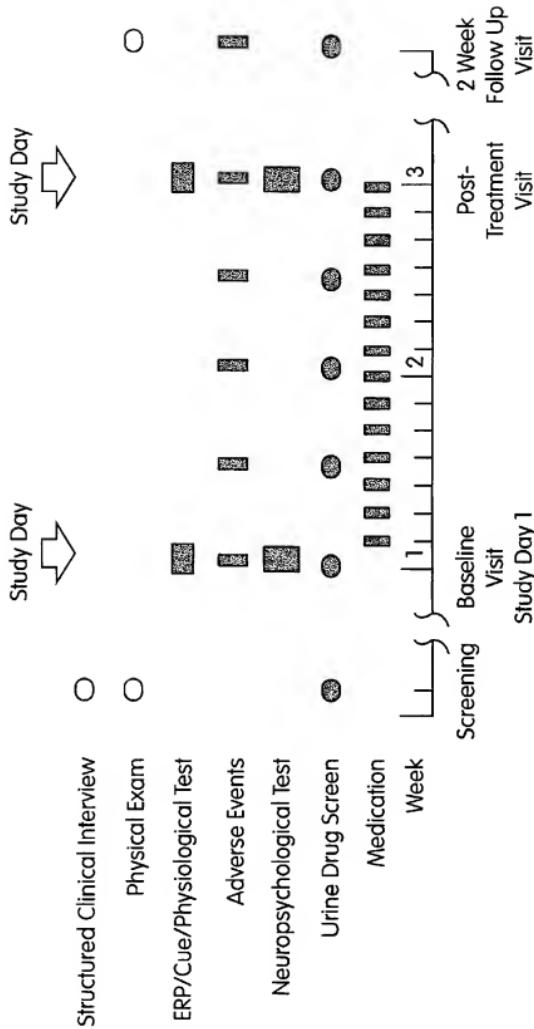
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1  
Fig.

## Likelihood to Use Cocaine

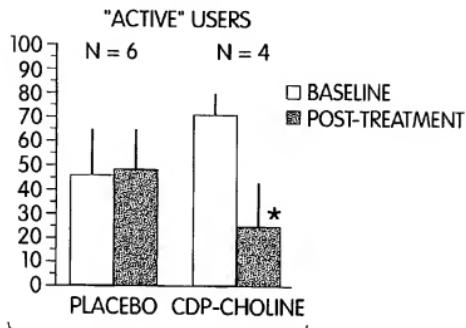


Fig. 2A

## Likelihood to Use Cocaine

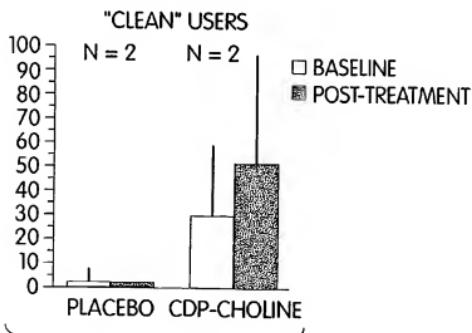
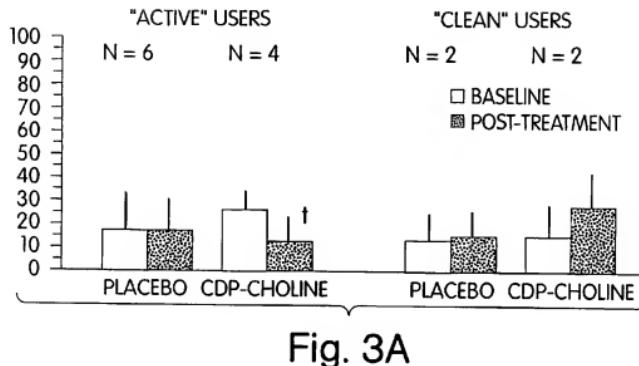


Fig. 2B

## Planning to Use Cocaine



## Desire to Use Cocaine

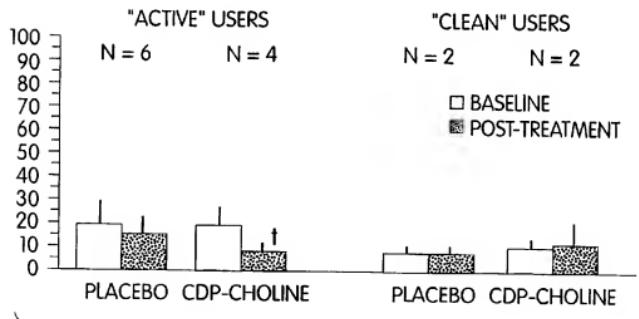


Fig. 3B

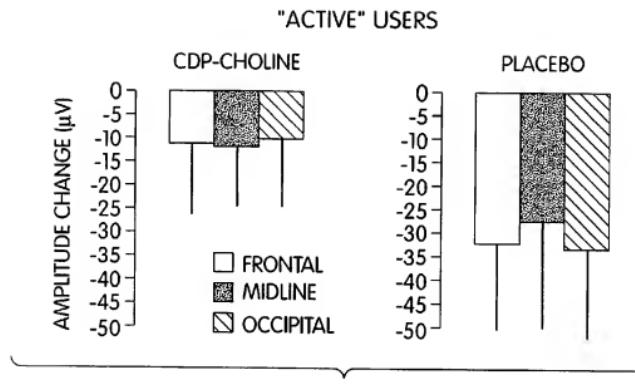


Fig. 4

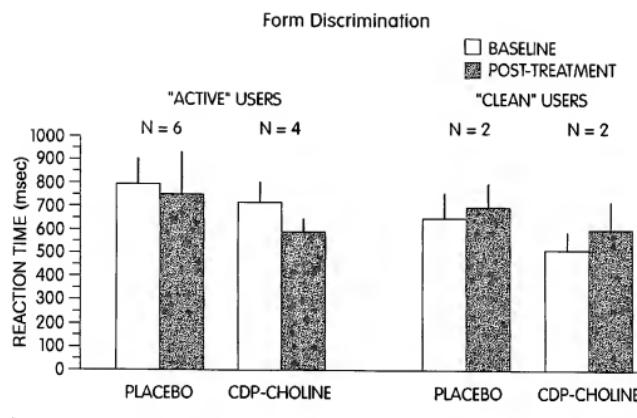
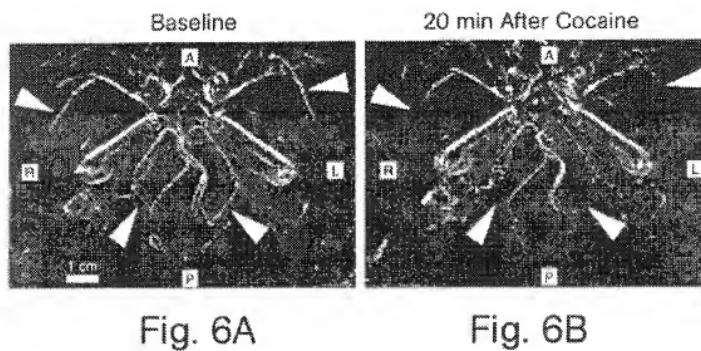


Fig. 5



## Effects of Cocaine or Placebo Administration on Cardiovascular Parameters

Variable	Placebo (n=7)	0.2 mg/kg Cocaine (n=9)	0.4 mg/kg Cocaine (n=8)	ANOVA $F_{2,21}$						
	Baseline	Peak + 20 min	Baseline							
HR	63 ± 3	69 ± 3 <sup>b</sup>	64 ± 2*	67 ± 2	105 ± 4 <sup>b</sup>	85 ± 2 <sup>cc</sup>	66 ± 3	112 ± 6 <sup>bb</sup>	98 ± 5 <sup>cc</sup>	15.0 <0.002
Sys	129 ± 8	142 ± 7 <sup>b</sup>	133 ± 8**	131 ± 5	152 ± 6 <sup>b</sup>	137 ± 5**	117 ± 4 <sup>a</sup>	147 ± 7 <sup>bb</sup>	98 ± 5 <sup>cc**</sup>	0.3 0.58
Dias	62 ± 3	71 ± 2 <sup>bb</sup>	66 ± 4	68 ± 3	89 ± 4 <sup>b</sup>	78 ± 4**	74 ± 3 <sup>aa</sup>	94 ± 6 <sup>bb</sup>	87 ± 4 <sup>cc</sup>	5.1 <0.05

Shown are means ± standard errors. ANOVA, repeated measures analysis of variance of heart rate, beats per minute; Sys and Dias, Systolic and Diastolic blood pressures, mm Hg.

a. Significantly different from baseline for 0.2 mg/kg dose group. (p < 0.03, unpaired t-test)

aa. Significantly different from baseline for placebo dose group. (p < 0.02, unpaired t-test)

b. Significantly different from baseline within dose group, paired t-test p < 0.01

bb. Significantly different from baseline within dose group, paired t-test p < 0.001

c. Significantly different from baseline within dose group, paired t-test p < 0.02

cc. Significantly different from baseline within dose group, paired t-test p < 0.002

\* Significantly different from peak within dose group, paired t-test p < 0.05

\*\* Significantly different from peak within dose group, paired t-test p < 0.005

Fig. 7

## Magnetic Resonance Angiography Results

Cocaine Dosage (mg/kg)	Angiography Rating			Discordant Rating
	Unchanged	Ambiguous	Altered	
0.0	4	0	1	2
0.2	3	1	3	2
0.4	2	1	5	0

Shown are numbers of cases for each MRA rating category and cocaine dosage level.

Fig. 8

Group: Measure:	Comparison (n = 16)	HMI Maintenance (n = 15)	HW Short Term Maintenance (n = 7)	HMI Long Term Maintenance (n = 8)	HMI vs. Comparison t unpaired t (df = 29)	HMI vs. Comparison t unpaired t (df = 21)	Long vs. Comparison t unpaired t (df = 22)	Short vs. Long unpaired t (df = 13)
%PME	8.9 ± 0.9	10.1 ± 1.4	10.8 ± 1.5	9.5 ± 1.1	p < 0.008	p < 0.002		
%PI	5.9 ± 0.8	5.7 ± 0.9	5.7 ± 0.8	5.7 ± 1.0				
%PDE	30.2 ± 2.3	33.2 ± 3.9	35.5 ± 4.0	31.2 ± 2.5	p < 0.02	p < 0.001		p < 0.03
%PCr	13.3 ± 0.9	11.5 ± 1.3	10.6 ± 1.0	12.3 ± 1.0	p = 0.0001	p < 0.0001	p < 0.03	p < 0.01
%β-NTP	10.7 ± 1.3	10.1 ± 1.3	10.1 ± 1.3	10.1 ± 1.3				
%Total NTP	41.8 ± 3.5	39.5 ± 4.3	37.4 ± 4.4	41.3 ± 3.5				
PCr/PI	2.3 ± 0.4	2.1 ± 0.6	1.9 ± 0.2	2.3 ± 0.4			p < 0.02	
β-NTP/PCr	0.81 ± 0.12	0.89 ± 0.16	0.96 ± 0.14	0.83 ± 0.16			p < 0.02	
PME/PDE	0.30 ± 0.02	0.30 ± 0.03	0.30 ± 0.02	0.30 ± 0.03				
pH	7.05 ± 0.02	7.05 ± 0.03	7.06 ± 0.03	7.04 ± 0.03				

Fig. 9

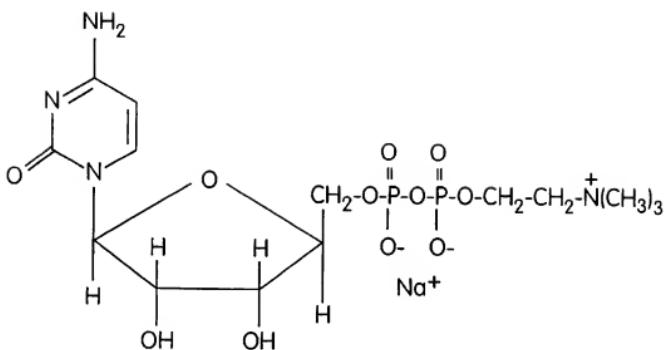


Fig. 10

**CYTIDINE-CONTAINING AND CYTOSINE-CONTAINING COMPOUNDS AS TREATMENTS FOR STIMULANT EXPOSURE**

This application is a continuation of Ser. No. 08/908,997 filed Aug. 8, 1997 now U.S. Pat. No. 5,958,896.

**BACKGROUND OF THE INVENTION**

This invention relates to methods for the treatment of stimulant abuse and addiction.

In the mid-1980's, the use of the stimulant cocaine reached epidemic levels in the United States, and even today abuse of this drug remains widespread: In 1995, the Substance Abuse and Mental Health Administration reported that nearly 2.5 million Americans admitted occasional and 600,000 admitted frequent cocaine use. The adverse societal and health consequences stemming from such cocaine use are significant. First, there is the hidden toll of emotionally and psychologically damaged families dealing with a family member having a cocaine dependency. And second, there is the adverse exposure to potentially detrimental health consequences associated with cocaine use and abuse.

Although historically the frequency of hospital admissions associated with cocaine abuse has been relatively low (0.35-3%), hospital visits stemming from a cocaine-related event now appear to be on the increase. In addition, the case report literature illustrating catastrophic neurological and cerebrovascular complications in cocaine users is also rapidly growing, and the incidence of cocaine-related strokes has been characterized as reaching epidemic proportions. Surprisingly, many cocaine-related deaths are associated with any major brain pathology upon autopsy, yet patients have been observed to show signs associated with moderate to severe cognitive dysfunction.

Moreover, experts in this area have observed that even during periods of cocaine abstinence cognitive abnormalities persist, suggesting that brain dysfunction occurs and is maintained beyond the period of acute cocaine intoxication. The "clinically silent" nature of these abnormalities implies that substantial numbers of cocaine users may be affected with deficits as yet undiagnosed. The etiologies of these subtle changes have not been elucidated, although cocaine-induced vasoconstriction and vasospasm have been implicated.

To date, there are no approved pharmacotherapies for cocaine abuse and dependence although the need for such therapies is clear.

**SUMMARY OF THE INVENTION**

In general, the invention features a method of treating a mammal exposed to a stimulant involving administering to the mammal, a therapeutically-effective amount of either a cytidine-containing or cytosine-containing compound.

In preferred embodiments, the mammal is a human; the stimulant is cocaine; the therapeutically effective compound is a cytidine-containing compound, for example, one that includes cytidine or CDP; the cytidine-containing or cytosine-containing compound further includes choline (and is, for example, CDP-choline); the mammal being treated has a stimulant dependency or stimulant craving; and the mammal being treated is a pregnant woman or a child with antenatal exposure to a stimulant.

In another aspect, the invention features a method for treating cerebral vasoconstriction sequelae in a mammal,

involving administering a therapeutically-effective amount of either a cytosine-containing or cytidine-containing compound to the mammal. In various preferred embodiments, the vasoconstriction is cocaine induced; the vasoconstriction is induced by a substance that causes vasoconstriction; the mammal is a human; the therapeutically effective compound is a cytidine-containing compound, for example, one that includes cytidine or CDP; and the cytidine-containing or cytosine-containing compound further includes choline (and is, for example, CDP-choline).

By "treating" is meant the medical management of a patient with the intent that a cure, amelioration, or prevention of a dependency or a relapse or associated disease, pathological condition, or disorder will result. This term includes active treatment, that is, treatment directed specifically toward improvement of the dependency or associated cure of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the dependency or associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the dependency, disease, pathological condition, or disorder; preventive treatment, that is, treatment directed prevention of the dependency or associated disease, pathological condition, or disorder; and supportive treatment that is, treatment employed to supplement another specific therapy directed toward the improvement of the dependency or associated disease, pathological condition, or disorder. The term "treating" also includes symptomatic treatment, that is, treatment directed toward constitutional symptoms of the dependency or an associated disease, pathological condition, or disorder.

By "exposure" and "exposed" is meant the condition of being subjected to a stimulant either inadvertently or intentionally. This term shall include any mechanism for introducing the stimulant to the mammal, the most typical being instillation, inhalation, and intravenous administration. This term also includes exposure to a stimulant when presented in combination with other compounds not considered stimulants. The term exposure may also represent single or multiple incidents.

By "stimulan" is meant any substance that temporarily increases functional activity, and preferably cardiac, respiratory, cerebral, nervous, vascular, motor, or vasomotor functional activity. Preferred stimulants include, without limitation, cocaine, amphetamines, methamphetamine, and methylphenidate.

By "therapeutically-effective amount" is meant an amount of a cytidine-containing or cytosine-containing compound sufficient to produce a healing, curative, or ameliorative effect either in the treatment of a stimulant exposure or stimulant dependency.

By "cytidine-containing compound" is meant any compound that includes, as a component, cytidine, CMP, CDP, CTP, dCMP, dCDP, or dCTP. Preferred cytidine-containing compounds include, without limitation, CDP-choline and cytidine 5'-diphosphocholine frequently prepared as cytidine 5'-diphosphocholine [sodium salt] and also known as citicoline.

By "cytosine-containing compound" is meant any compound that includes, as a component, cytosine.

By "dependency" is meant any form of behavior that indicates an altered or reduced ability to make decisions resulting, at least in part, from the use of stimulants. Representative forms of dependency behavior may take the form

of antisocial, inappropriate, or illegal behavior and include those behaviors directed at the desire, planning, acquiring, and use of stimulants. This term also includes the psychic craving for a drug that may or may not be accompanied by a physiological dependency, as well as a state in which there is a compulsion to take a drug, either continuously or periodically, in order to experience its psychic effects or to avoid the discomfort of its absence. Forms of "dependency" include habituation; that is, an emotional or psychological dependence on a compound to obtain relief from tension and emotional discomfort, as well as physical or physiological dependence, that is, use of a compound to prevent withdrawal symptoms.

By "antepartal exposure" is meant exposure of a subject to a stimulant before birth via the antepartal mother, the antepartal mother having had an exposure as described herein.

By "craving" is meant a behavior that reflects a consuming desire, longing, or yearning for a stimulant. This term may refer to aspects of behaviors that are components of a dependency.

By "cerebral vasoconstriction sequelae" is meant any condition following and resulting from the constriction of blood vessels in the cerebrum provoked by a motor nerve or chemical compound, for example, any disease, pathology, disorder, or dependency subsequent to stimulant exposure. This term includes cerebral ischemia, neuropathologies, neurological deficits, altered brain chemistry, reduced levels of task mastering, cognitive impairment, behavioral changes, vegetative responses, mental deterioration, altered conditioned avoidance and auditory response parameters, and motor activity impairment. Such conditions may be characterized by altered levels of phosphomonoesters (PME), phosphodiesters (PDE), phosphocreatine (PCr), nucleotide triphosphates (NTP), inorganic phosphorus (Pi), the PCr/Pi ratio, the  $\beta$ -NTP/PCr ratio, cerebral phosphorus metabolites, phospholipid precursors, cellular and organelle phospholipid synthesis, membrane synthesis, tyrosine hydroxylase activity, dopamine and dopamine metabolism, bioenergetic function, fatty acid release, neutral acids, phosphatidylcholine and glycerophospholipid degradation, glucose, pyruvate, acetylcholine, norepinephrine, vasodilation, synaptosomal phosphorylation, cellular proliferation, neuronal injury, edema, mitochondrial ATPase and  $Na^+ - K^+$  ATPase sensitivity, phospholipase A<sub>2</sub> activation, EEG parameters, cardiovascular and respiratory parameters. The term shall include any of the above conditions altered alone or in combination.

The present invention provides a number of advantages. Importantly, it provides one of the first therapeutics for the treatment of stimulant dependencies (such as cocaine dependencies). In addition, the cytidine-containing compounds utilized herein are relatively non-toxic, and CDP-choline, in particular, is pharmacokinetically understood and known to be well tolerated by mammals.

#### DETAILED DESCRIPTION OF THE INVENTION

The drawings will first briefly be described.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is diagram depicting a time line and the points at which various procedures were performed throughout the experimental protocol described herein.

FIGS. 2A and 2B are histograms depicting changes in the likelihood to use cocaine in patients classified as "active"

(FIG. 2A) versus "clean" (FIG. 2B) when treated with CDP-choline (\* indicates significance at p<0.05 relative to baseline). The unshaded bars represent patients at baseline, and the shaded bars represent patients post-treatment. The y axis is a visual analog scale (VAS) in millimeters where 100 represents "most ever" and zero represents "not at all." The VAS is designed to quantitate subjective mood statements.

FIGS. 3A and 3B are histograms depicting changes in the percent levels of cocaine craving exhibited by patients when treated with CDP-choline (# indicates a trend relative to baseline). The unshaded bars represent patients at baseline, and the shaded bars represent patients post-treatment. The scale is the same as described in FIGS. 2A and 2B.

FIG. 4 is a histogram depicting improved cognitive processing ability in patients who are "active" users treated with CDP-choline as measured in the P300 ERP test.

FIG. 5 is a histogram depicting iconic memory performance in patients classified as "active" when treated with CDP-choline. The unshaded bars represent patients at baseline, and the shaded bars represent patients post-treatment.

FIGS. 6A and 6B are Magnetic Resonance Images (MRI) depicting marked cerebral vasoconstriction following intravenous cocaine administration. Axial maximum intensity projection images are indicated at baseline (FIG. 6A) and 20 minutes following intravenous cocaine (0.4 mg/kg) administration (FIG. 6B). Cocaine induced a signal loss at distal segments of the middle cerebral arteries (upper arrowheads) and in the posterior cerebral arteries (lower arrowheads), indicative of vasoconstriction. A=anterior, P=posterior, L=left, and R=right.

FIG. 7 is a chart depicting the effects of cocaine on cardiovascular parameters such as heart rate (HR) and systolic (Sys) and diastolic (Dia) blood pressures.

FIG. 8 is a chart depicting the effects of cocaine on cerebral vasoconstriction as measured by magnetic resonance angiography (MRA).

FIG. 9 is a chart depicting percent differences in cerebral phosphorus metabolite profiles of patients on methadone maintenance (MM) as measured by phosphorus magnetic resonance spectroscopy.

FIG. 10 is a depiction of the molecular structure of CDP-choline [sodium salt], also known as citicoline.

The invention described herein features a method for the treatment of stimulant abuse and its symptoms, as well as stimulant dependency and associated self-destructive behaviors. The invention focuses on cocaine abuse and addiction although other stimulant dependencies may be similarly treated. To this end, the invention features the use of cytidine-containing or cytosine-containing compounds to alleviate symptoms of abuse and dependency. One preferred cytidine-containing compound is CDP-choline (also referred to as citicoline or CDP choline [sodium salt]). As described herein, CDP-choline has been found to have two important therapeutic properties. First, CDP-choline improves brain chemistry in patients suffering from symptoms of cocaine abuse as a result of cocaine-induced cerebral vasoconstriction. And second, CDP-choline alleviates the dependency exhibited by active cocaine users.

In addition, the results described herein demonstrate that symptoms of cocaine abuse are very likely functions of cocaine-induced cerebral vasoconstriction. These results also demonstrate that symptoms of opiate abuse and dependency (specifically, heroin abuse and dependency) induce alterations in some, but not all, brain chemistry indices and

that certain of those parameters in patients with heroin addictions are improved with methadone in a manner akin to improvements observed in patients with cocaine addictions treated with CDP-choline. The present invention therefore enables methods and reagents for the treatment of stimulant abuse, such as cocaine abuse, by providing original data from human trials.

The following detailed examples are provided for the purpose of illustrating, and not limiting, the invention.

#### EXAMPLE 1

##### CDP-Choline is an Effective Treatment for Cocaine Abuse and Dependence

A small double-blind clinical trial of CDP-choline versus placebo for altering cocaine craving and modifying responses to cocaine-related stimuli was conducted. A total of fourteen crack cocaine users were recruited by passing a psychiatric, medical, and clinical laboratory evaluation and provided informed consent to participate in this outpatient treatment study. The subjects were completely randomized and the resultant demographic profiles are shown in Table 1.

TABLE 1

Demographics of Study Population		
Variables	CDP-Choline*	Placebo
# of Subjects	6	8
Race (Cau/Afr Amer)	2/4	4/4
Active Users	4	6
Clean 6-12 months	2	2
Age	38.0 ± 6.1	35.8 ± 8.5
Weight (kg)	70.4 ± 6.5	86.7 ± 12.9
Sex (M/F)	9/2	7/1
Cocaine Use (yr)	9.8 ± 4.2	11.6 ± 3.9
Cocaine Use (#/week)	10.0 ± 10.0	7.2 ± 6.6
Ethanol Use (yr)	20.8 ± 6.9	18.4 ± 7.2
Ethanol Use (#/week)	13.0 ± 8.5	11.0 ± 4.7

\*500 mg oral dose twice daily

The subjects participated in two evaluation sessions, each separated by two weeks, during which subjects received either placebo or CDP-choline (500 mg, twice daily). Frequent assessments for adverse effects and urine screens were performed during treatment. The overall research design is depicted in FIG. 1.

On each of two assessments sessions, subjects were required to report to the laboratory to fill out a number of subjective mood state questionnaires. After this initial assessment, subjects then sat in a sound- and light-attenuated room and were prepared for standard EEG/ERP recording and physiological monitoring. After a 1 hour baseline period the first of three videos was shown. Subsequent videos were shown at 1 hour intervals. The three videos included: (1) a neutral tape of coral sea life; (2) an emotionally laden footage of the movie "An American Werewolf in London"; and (3) footage of two men buying, preparing, and smoking crack cocaine. Continuous physiologic and electrophysiologic brain mapping measures were made before and after each videotape.

In addition, subjects were required to answer a series of questionnaires were designed to assess the subject's degree of cocaine craving. These included questions like; "What is the likelihood of your using cocaine?"; "Are you planning to use cocaine?"; and "How much do you desire to use cocaine?". Finally, a test battery for reaction time and psychomotor function was given. The CalCAP™ is a series of 10 different tasks each becoming more difficult as the test proceeds.

#### Safety Assessments

The results of the assessments for adverse reactions revealed that CDP-choline not only had no side effects, but subjects were unable to detect whether they had received an active or placebo dose. No changes in health status or blood or urine chemistry analyses were observed. At baseline, three subjects in the placebo group and one in the CDP-choline group had nonclinical sinus bradycardia. One subject who received CDP-choline, developed a mild, nonrelevant increase in the cardiac P-R interval. Finally, there were no changes in heart rate or blood pressure.

#### Population Dynamics

A post-hoc analysis revealed that both the placebo and CDP-choline group contained two distinct populations of crack cocaine users. "Active" users were defined as those who were currently using cocaine at the time of recruitment. These reports were confirmed using urine drug screens. "Clean" users were those who had been cocaine-free for the past 6-12 months. The distribution of active and clean users is depicted in Table 2.

TABLE 2

Distribution of "Active" versus "Clean" Users in the Study Population			
	Treatment		
	Status	CDP-choline	Placebo
Active	4	6	6
Clean	2	2	2

This distinction appeared to be very important in characterizing the efficacy of CDP-choline.

#### Assessment of Cocaine Dependency/Craving

Responses to the Pre-Questionnaires with respect to "Likelihood to use cocaine" revealed a statistically significant decrease in this variable in the active cocaine users who had been treated with CDP-choline as compared to those patients treated with a placebo (FIGS. 2A and 2B).

As another measure of whether treatment with CDP-choline could decrease cocaine dependency in "active" versus "clean" users, patients were queried on the probability of their "planning to use cocaine" and their "desire to use cocaine" (FIGS. 3A and 3B). Although not statistically significant, there were strong trends ( $p=0.06-0.08$ ) for reductions in these parameters in "active" users. A similar reduction in the "likelihood to use" question was observed even after patients were subjected to the video depicting crack cocaine use (data not shown).

To test for the ability of CDP-choline to improve cognitive function in cocaine users, a P300 ERP test was performed on "active users" (FIG. 4). The P300 ERP test is a measure of memory encoding performance during the assessment of a novel stimulus. Reductions in amplitude are generally interpreted as reflecting reduced cognitive processing. FIG. 4 shows that (compared to baseline) the reduction in P300 amplitude was modest and insignificant in the CDP-choline treated "active" user while the reduction in placebo-treated "active" users was significantly greater.

To further test for the ability of CDP-choline to improve cognitive function in cocaine users, patients treated or untreated with CDP-choline were tested for their ability to discriminate forms using the CalCAP™ test (FIG. 6). Form discrimination was measured by showing the subjects 3

geometric shapes simultaneously and asking them to press a key when two were identical in shape and color. This task required that the subjects make rapid comparisons and measured their ability to retain iconic memory. Patients classified as "active" users who were treated with CDP-choline showed a trend toward significant improvement in their reaction time (145 msec) in Form Discrimination as compared to untreated "active" users (FIG. 5).

#### Summary of Human Clinical Trial Results

In this human trial designed to test the effectiveness of CDP-choline in the treatment of cocaine abuse and dependency, the following observations were made:

1. CDP-choline improved short term memory and cognitive function in patients with a cocaine exposure.
2. Patients with a cocaine dependency or craving were benefited by treatment with CDP-choline.
3. CDP-choline is nontoxic, well-tolerated, and was undetectable by the subjects.

These results indicate that CDP-choline is a useful adjunct to current therapies for cocaine abuse, particularly in individuals who are currently active users. Without being bound to a particular theory, these results are likely due to CDP-choline's weak dopamine agonist activity. In addition, these results indicate that, because of its low toxicity, CDP-choline may be useful for treating pregnant women, adolescents, and babies born to cocaine-dependent women. This last group is of particular importance because CDP-choline's ability to reduce stroke symptoms suggests that this therapeutic may also reverse many of the detrimental micro-infarcts that occur during *in utero* exposure to cocaine.

To date, there are no approved pharmacotherapies for cocaine abuse or dependence. Administering CDP-choline therapeutically therefore provides an important approach to minimizing the detrimental effects of cocaine abuse and dependency and to speeding the recovery process.

In addition, based on CDP-choline's mechanism of action, this and other cytidine-containing or cytosine-containing compounds are generally useful for the treatment of other types of stimulant abuse and dependence, including, but not limited to, amphetamine, methamphetamine, and methylphenidate abuse and dependence.

#### EXAMPLE 2

##### Cocaine-Induced Cerebral Vasoconstriction in Humans

This clinical study was designed to evaluate whether intravenous administration of low doses of pure, pharmaceutical grade cocaine hydrochloride could induce cerebral vasoconstriction in otherwise healthy human subjects. Serial noninvasive imaging of the major cerebral arteries was conducted at baseline and twenty minutes following cocaine administration, using magnetic resonance angiography (MRA). MRA is highly sensitive to blood flow perturbations. Vasoconstriction results in vessel signal intensity loss at the site of and distal to the constricted region, and MRA has proven useful for detecting acute cerebral vasospasm. This technique is noninvasive and does not utilize ionizing radiation, facilitating within-subject repeated-measure study designs.

##### Subjects

Subjects with either no history of cocaine use or with a diagnosis of cocaine abuse or dependence were excluded

from this study. A group of 24 healthy, medically and neurologically normal males aged 29±5 years (mean±SD) who reported casual cocaine use (median=8, range=3 to greater than 40 lifetime exposures, primarily via insufflation) was selected for study participation. Subjects provided written Informed Consent with McLean Hospital Institutional Review Board approval. Subjects underwent a complete physical and neurological exam including ECG and hematology prior to study, and provided a medical history including estimates of illicit drug usage. On the study day, subjects provided breath and urine samples to detect recent alcohol or illicit drug use. Breath samples were analyzed with an Alco Sensor III Breathalyser (Intoximeters Inc., St. Louis, Mo.). Urine samples were analyzed for the presence of cocaine, amphetamines, phencyclidine, opiates, barbiturates, benzodiazepines, and tetrahydrocannabinol with a Triage™ Test (Biosite Diagnostics, San Diego, Calif.). All subjects had negative breath alcohol samples and urine screens. Each subject had an 18G angiocath inserted in a vein overlying the antecubital fossa for cocaine or placebo administration. Subjects were fitted with noninvasive cardiovascular monitoring equipment (In Vivo Research, Inc., Orlando, Fla.) including 4 lead electrocardiogram (ECG), blood pressure cuff, and pulse oximeter, to provide continuous monitoring of ECG, blood pressure, and heart rate.

##### Magnetic Resonance Scanning

Magnetic resonance imaging was conducted with a 1.5 Tesla Sigma Scanner (General Electric, Milwaukee, Wis.). T1 weighted sagittal localizer images (TE/TR: 19/600 msec) were used to position MRA imaging sets. Angiogram imaging sets of 60 axial images were collected with the three dimensional Time of Flight (3D TOF) magnetization transfer imaging option with flow compensation and saturation. The following acquisition parameters were used: TE/TR: 35/20 msec, FOV: 19 cm, matrix: 256x192, slice thickness: 1.2 mm, 1 NEX, imaging time: 7.5 minutes. Each image set produced a single axial maximum intensity projection (MIP) image which was analyzed for drug effects. Cocaine (0.2 or 0.4 mg/kg) or placebo was then administered by slow intravenous injection over 1 minute; all doses were given in a double-blind manner. Seventeen minutes after drug administration, a post-drug 3D TOF series was initiated, with a midpoint of the imaging sequence occurring 20 minutes after drug administration.

##### Image Analysis

Baseline and post cocaine/placebo axial MIP images from each subject were analyzed for drug-induced changes. Two expert raters, blinded with regard to study drug administration, independently analyzed the 24 image sets. Prior to analysis, the 2 raters agreed on criteria that would be used to determine alterations between baseline and post-drug images. Subtle image differences discernible by both raters, including change in the caliber of moderate and large sized arteries and focal narrowing or complete signal loss in a major arterial structure were considered as alterations. Image sets were scored as unchanged, ambiguous, or altered. Concordance was established when both raters agreed in their independent scan ratings. A weighted kappa statistic of 0.64 for interrater agreement showed a very high degree of between-rater concordance ( $p=0.002$ , two-sided; unweighted kappa=0.70,  $p=0.0001$ ) (Fleiss, J L, et al, *Educ. Psychol. Meas.*, 33:613-619, 1973).

##### Results of Study on Cocaine-Induced Cerebral Vasoconstriction

Baseline cardiovascular parameters were normal in all subjects, with heart rate (HR) averaging 68±2 bpm

(mean $\pm$ SE), and systolic (Sys) and diastolic (Dias) blood pressures averaging  $126\pm 3$  and  $70\pm 3$  mm Hg, respectively (FIG. 7). Slight increases in HR, Sys, and Dias were observed in the placebo-administered group (FIG. 7) and were attributed to expectancy effects. Both cocaine doses elevated heart rate for the duration of the experiment, with peak increases in HR, Sys, and Dias (FIG. 7) occurring approximately 6–10 minutes following drug administration. Twenty minutes after cocaine or placebo administration, at the midpoint of the MRA acquisition, HR and Dias remained elevated in all subjects administered cocaine and Sys remained elevated in subjects administered 0.4 mg/kg cocaine (FIG. 7). An overall dose effect of cocaine (repeated measures ANOVA) was detected for HR and Dias at the 20 minute time point (FIG. 7).

Image analysis revealed a relationship between cocaine administration and angiographic alteration. All baseline images were judged to be normal. Raters determined that 5 of 8 subjects who received 0.4 mg/kg cocaine experienced angiographic alterations indicative of cerebral vasoconstriction. These ranged from subtle differences in arterial caliber to more significant alterations, including focal narrowing or complete signal loss from a major arterial structure. These alterations were detected in the posterior cerebral artery, the middle cerebral arteries (FIG. 6), vertebral arteries, and the anterior and posterior communicating arteries. Three of 9 subjects who received 0.2 mg/kg cocaine had angiographic alterations in several arteries including the anterior communicating arteries and the posterior and middle cerebral arteries. One of 7 subjects who received placebo was ruled to have an altered post-placebo MRA scan. FIG. 8 shows the observed classification of angiogram results stratified by cocaine dosage for all image sets. Statistical analysis of discordantly rated scans using a linear-by-linear association model (Agresti, A, Categorical Data Analysis. John Wiley & Sons, New York, 1990) for the ordered categories of 0=unchanged, 1=ambiguous, and 2=altered, indicated a significant association of increasing prevalence of altered scans with increasing cocaine dose ( $p<0.041$ , one-sided). When discordantly rated scans were included, the significance of the association decreased slightly ( $p=0.056$ ). These findings demonstrated an apparent relationship between cocaine administration and altered MRA scan; moreover, this effect appeared to be dose-related. A stratified analysis of this small sample by frequency of self-reported lifetime cocaine use (1–10 times, 11–40 times, or greater than 40 times) revealed a statistically stronger dose-response relationship ( $p<0.001$ ), suggesting that prior cocaine use may have a cumulative effect in promoting angiographic changes indicative of vasoconstriction.

The study design precluded direct measurement of plasma cocaine levels in the present study population. However, we obtained plasma cocaine levels by gas chromatographic analysis (Teoh, S K et al, *J. Clin. Psychopharmacology* 13:87–99, 1993) from comparable subjects administered cocaine by identical protocols. Peak plasma cocaine levels of  $230\pm 10$  and  $90\pm 10$  ng/ml were found 6–8 minutes following intravenous administration of 0.4 ( $n=3$ ) and 0.2 ( $n=6$ ) mg/kg doses of cocaine, respectively. Plasma cocaine levels of  $180\pm 30$  and  $80\pm 10$  ng/ml were found at 20 minutes post-administration, corresponding to the midpoint time of the present MRA acquisition, following 0.4 and 0.2 mg/kg cocaine doses, respectively. These values and their course closely parallel those published in a recent report of the venous plasma cocaine level time course following intravenous cocaine administration (Evans, S M et al, *J. Pharmacol. Exper. Therap.* 279:1345–1356, 1996).

## Summary of Results

The above results are the first to document that intravenous administration of a relatively low dose of cocaine to otherwise healthy humans can induce angiographic changes indicative of cerebral vasoconstriction. This finding suggests that low cocaine doses are sufficient to induce cerebrovascular dysfunction. The data also reflects a dose-effect relationship between cocaine and vasoconstriction. This finding suggests that moderate to heavy cocaine users, who may attain plasma cocaine levels greatly exceeding those likely to have been achieved in this study, may experience a higher incidence of cerebral vasoconstriction. As cerebral vasoconstriction has been linked to hypoperfusion and persistent hypoperfusion has been associated with neuronal dysfunction, the present findings indicate that moderate to heavy cocaine use is likely associated with neuronal damage.

## Cumulative Effects of Cocaine and the Etiology of Chronic Cocaine-Induced Brain Dysfunction

Although it is assumed that chronic cocaine abuse is required to produce persistent perfusion deficits and cognitive dysfunction, it is presently unclear what threshold level of cocaine exposure results in these conditions. The cognitive dysfunction observed in chronic cocaine abusers is related to amount of cocaine used, suggesting a cumulative effect of cocaine on brain function. The present study documents a relationship between prior cocaine use and the propensity to experience vasoconstriction, suggesting that cocaine may have a cumulative effect in producing cerebrovascular dysfunction in addition to its acute vasoconstrictive effect. In this regard, self-reported lifetime cocaine use of more than 10 times nearly doubled the risk for experiencing a cocaine-induced angiographic change (75%) compared to the risk experienced by subjects reporting 10 or fewer episodes of lifetime cocaine use (38%). The present data suggest that the incidence of cocaine-induced cerebral vasoconstriction may be increased in individuals who escalate from experimental to casual or recreational cocaine use.

The present study was conducted at a single time point following cocaine administration, precluding analysis of the time-dependence of cocaine-induced vasoconstriction. Because cocaine-induced vasoconstriction is a transient phenomenon and because our time frame for its detection was short, it is conceivable that more subjects experienced vasoconstriction than detected in the current study. Additionally, we are unable to address whether cocaine or its metabolites, some of which are potent vasoconstrictors, mediate vasoconstriction. Cocaine metabolites may play an important role in inducing delayed cerebral vasoconstriction, because their levels gradually increase over several hours, and in extreme cases persist for up to several weeks. Thus they may trigger prolonged cerebral vasoconstriction associated with decreased cerebral perfusion.

The present study used intravenous cocaine administration as the drug delivery method, while intranasal administration and smoking of the alkaloidal form "crack" are the more common forms of administration. The mode of cocaine administration has been suggested to be related to cerebrovascular effect, with the intravenous route leading to hemorrhagic strokes and "crack" smoking leading to both ischemic and hemorrhagic stroke. Thus, it is possible that different forms of cocaine or different routes of administration may produce dissimilar rates or severity of vasoconstriction. However, our finding of a dose-effect relationship

between cocaine and vasoconstriction suggests that once a sufficient plasma cocaine concentration is achieved, cerebral vasoconstriction likely occurs.

Moreover, the results of this clinical trial demonstrate a real and substantial dose-effect relationship between cocaine and cerebral vasoconstriction. These results underscore the risks of single doses of cocaine in promoting cerebrovascular abnormalities, particularly in individuals with other risk factors. The data also strongly suggest that there is an increased risk of cerebrovascular dysfunction in individuals who are frequent or chronic cocaine users, and that this dysfunction may be progressive. Together, these findings highlight the potential dangers of cocaine use on cerebrovascular function and document the importance of cytosine-containing or cytidine-containing treatments such as CDP-choline that protect against or correct vasoconstriction or its symptoms.

#### EXAMPLE 3

##### Cerebral Phosphorus Metabolism in Heroin-Dependent Polydrug Abusers During Methadone Maintenance

Heroin abusers have cerebral metabolic and perfusion abnormalities that persist beyond the period of drug intoxication and acute withdrawal (London, E D, et al. *Res. Comm. Subst. Abuse* 10:141-144, 1989; Rose, J S et al., *Psychiatry Research: Neuroimaging* 67:39-47, 1996). A number of opiates, including candidates for the treatment of opiate abuse, have been evaluated for their effects on brain function (London, E D, et al. *Res. Comm. Subst. Abuse* 10:141-144, 1989; London, E D et al. *Arch. Gen. Psychiatry* 47:73-81, 1990; Walsh, S L et al. *Neuropsychopharmacology* 10:157-170, 1994). However, no study to date has examined the neurochemical effects of the most widely utilized intervention for the treatment of opiate abuse, methadone. Methadone has demonstrated efficacy in improving psychiatric symptoms and overall health in opiate abusers (McLellan, A T et al. *JAMA* 247:1423-1428, 1982; Ball, J C et al. *The effectiveness of methadone maintenance treatment: Patients, programs, services, and outcome*. New York, Springer-Verlag, 1991). However, it is unknown whether these clinical improvements are related to improved cerebral function. Consequently, the present study was conducted in heroin-dependent polydrug abusers chronically treated with methadone, to evaluate indices of cerebral biochemistry using phosphorus magnetic resonance spectroscopy ( $^{31}\text{P}$  MRS).  $^{31}\text{P}$  MRS allows noninvasive measurement of brain membrane integrity and bioenergetic status.

To carry out this clinical study, heroin-dependent polydrug abusers (9 females and 6 males, aged 40±4 years,  $\text{mean} \pm \text{SD}$ ) were recruited from an outpatient methadone maintenance (MM) clinic. Potential subjects with a history of alcohol abuse or HIV infection were excluded from the study. This population was subdivided into two groups based on MM treatment duration: the short-term group ( $n=7$ , 40±24 weeks) and the long-term group ( $n=8$ , 137±53 weeks). The methadone dose administered to these subjects was in the range of 60-80 mg per day and was statistically equivalent in the two MM treatment duration groups. All medical histories, including random biweekly urine toxicology testing throughout the course of methadone maintenance therapy, were reviewed in order to determine study population demographics. Positive urine tests for the following substances were found greater than 10% of the time: methadone—98%; benzodiazepines—39%; opiates—37%; cocaine—18%. Clean urine screens were found 26% of the

time. These frequencies were statistically equivalent across MM duration subgroups.

An age matched comparison group (6 females and 10 males, aged 40±4 years) with no history of substance abuse or neurological or psychiatric disorder was studied with identical procedures. All subjects provided written informed consent with McLean Hospital Institutional Review Board of Approval.

All subjects provided breath and urine samples immediately prior to scanning to determine whether recent alcohol or illicit drug use had occurred. Breath samples were analyzed with an Alco Sensor III Breathalyzer (Intoximeters Inc., St. Louis, Mo.). A positive breath alcohol sample was grounds for study exclusion. Urine samples were analyzed for the presence of illicit drugs with a Triage<sup>TM</sup> Test (Biosite Diagnostics, San Diego, Calif.). A positive urine test for illicit drug use was not grounds for exclusion in the MM population as it was assumed that this group would have ongoing drug use. The frequency of positive Triage testing for all substances was statistically equivalent across MM duration subgroups.

#### Imaging

Spectra were acquired on a 1.5 Tesla General Electric Signa Scanner using a doubly-tuned, linear proton, quadrature phosphorus head coil. An axial whole brain slice volume of 50 mm thickness was prescribed through the orbitofrontal/occipital cortices as described (Christensen, J D et al. *Magn. Reson. Med.* 35:658-663, 1996).

Spectra were processed with VARPROM/RUI (van den Boogaart, A et al. *NMR Biomed.* 8:87-93, 1995). A 5 Hz exponential line broadening filter was applied, and 7 peaks were fit to gaussian lineshapes by automated fitting: phosphomonoesters (PME), inorganic phosphorus (Pi), phosphodiesters (PDE), phosphocreatine (PCr), and  $\gamma$ ,  $\alpha$ , and  $\beta$ -nucleoside phosphates. The total phosphorus signal (summation of all peak areas) was statistically equivalent across groups allowing use of the % metabolite measure, the ratio of the area of each metabolite peak divided by the total phosphorus area, for between-group comparisons (Klunk, W et al. *Neurobiol. Aging* 15:133-140, 1994). Statistical analyses were performed using unpaired two-sided t-tests.

#### Results

The mole percentages of PME and PDE were significantly higher and the mole percent PCr was significantly reduced in the MM population (FIG. 9). When stratified into the short- and long-term MM treatment subgroups, different profiles of cerebral phosphorus metabolite abnormalities emerged. The short-term MM group had elevated % PME, % PDE, and  $\beta$ -NTP/PCr ratio, and reduced % PCr, % NTP, and PCr/Pi ratio. In contrast, the long-term MM group differed from the healthy comparison group only in having reduced % PCr levels. This group differed from the short-term MM group having higher % PCr levels and lower % PDE levels (FIG. 9).

#### Summary

The above findings indicated elevated PME and PDE as well as decreased PCr levels in heroin-dependent polydrug abusers. This may reflect membrane dysfunction and oxidative metabolism impairment secondary to perfusion defects. The metabolite profile is unique compared to findings in cocaine abusers in whom only PME and PDE elevations were noted and in cocaine-dependent polydrug

abusers in whom elevated PME and decreased  $\beta$ -NTP levels were found. This suggests that polydrug abuse populations with different primary substance abuse patterns may have discrete phosphorus metabolite profiles.

The present data are quite interesting in that they also document an apparent normalization of most cerebral phosphorus metabolites in subjects who have undergone prolonged MM therapy. In this regard, only PCr levels were abnormal in the long-term MM group; PCr and PDE levels were significantly higher and lower (more normal) in this versus the short-term MM group. This apparent improvement is consistent with a prior study documenting improved cerebral perfusion in short-term abstinence from heroin abuse (Resc, J S et al, *Psychiatry Research: Neuroimaging* 67:39-47, 1996). What is further encouraging from the present data is that metabolic improvements were noted in subjects with ongoing illicit substance use (opiate positive urines nearly 40% of the time). This indicates that abstinence is not required for normalization of certain aspects of brain function. Together, the present findings suggest that cerebral phosphorus metabolites are useful markers of brain health and treatment efficacy in individuals with a polydrug abuse history. As also indicated by the above study, certain aspects of altered phosphorus metabolism in heroin addicts are unique compared to findings in cocaine abusers. Other aspects of this altered metabolism, such as membrane dysfunction and oxidative metabolism impairment secondary to perfusion defects, are common to both drug abusers and may therefore be treatable with cytosine-containing or cytidine-containing compounds, such as CDP-choline.

#### EXAMPLE 4

##### Cytidine-Containing and Cytosine-Containing Compounds

The human trials described herein made exclusive use of the cytidine-containing compound, CDP-choline, also known as citicoline, received from Interneuron Pharmaceuticals Inc. Nonetheless, because the cytidine moiety of this compound is responsible for the beneficial effects observed in these trials, any of a variety of cytidine-containing or cytosine-containing compounds are suitable for the treatment of the afflictions described herein. Examples of useful cytidine-containing or cytosine-containing compounds may include any compound comprising one of the following: cytosine, cytidine, CMP, CTP, dCMP, dCDP, and dCTP. Preferred cytidine-containing compounds include CDP-choline and cytidine 5'-diphosphocholine [sodium salt]. The above list of cytidine-containing and cytosine-containing compounds is provided to illustrate, rather than to limit the invention, and the compounds described above are commercially available, for example, from Sigma Chemical Company (St. Louis, Mo.). The molecular structure of CDP-choline [sodium salt] is provided in FIG. 10.

As noted above, one particular source of CDP-choline is Interneuron Pharmaceutical, Inc. The compound obtained from this source has the following characteristics:

Chemical Formula:  $C_{14}H_{28}N_4O_{11}P_2Na$

Molecular Weight: 510.31

Physical and Chemical Characteristics: completely soluble in water as a 10% solution; practically insoluble in 100% ethanol.

The pH in water is between 6.5-7.5.

An available clinical dosage form of CDP-choline for oral administration is a 500 mg oblong tablet. Each tablet contains 522.5 mg CDP-choline sodium, equivalent to 500 mg

of CDP-choline. Matching placebo tablets are also available. The excipients contained in both active and placebo tablets are talc, magnesium stearate, colloidal silicon dioxide, hydrogenated castor oil, sodium carboxy-methylcellulose, and microcrystalline cellulose.

CDP-choline is a naturally occurring compound that is synthesized from cytidine-5'-triphosphate and phosphocholine with accompanying production of inorganic pyrophosphate in a reversible reaction catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (Weiss, Metabolism and Actions of CDP-choline as an Exogenous Compound and Administered Exogenously as Citicoline, *Life Sciences* 56:637-660, 1995).

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#### EXAMPLE 5

##### Administration of Cytidine-Containing and Cytosine-Containing Compounds

Cytidine-containing and cytosine-containing compounds, such as CDP-choline, are naturally occurring endogenous compounds. CDP-choline itself is synthesized in a sodium salt form for clinical use (see FIG. 10). One clinical dosage form for oral administration is a 500 mg oblong tablet. Each tablet contains 522.5 mg of CDP-choline sodium, equivalent to 500 mg of CDP-choline. For easier administration, such tablets may be cut into smaller pieces or crushed. Preferably, cytosine-containing and cytidine-containing compounds, such as CDP-choline, are administered at a dosage of at least 500 mg twice daily by oral administration.

30 Orally administered CDP-choline is bioavailable, with more than 99% of CDP-choline and/or its metabolites absorbed and less than 1% excreted in feces. CDP-choline, administered either orally or intravenously, is rapidly converted into the two major circulating metabolites, choline and cytidine. Major excretion routes are lung (12.9%) and urine (2.4%); the rest of the dose (83.9%) is apparently metabolized and retained in tissues.

Other formulations for treatment or prevention of the afflictions described herein, may take the form of a cytosine-containing or cytidine-containing compound, such as CDP-choline, combined with a pharmaceutically-acceptable diluent, carrier, stabilizer, or excipient. Conventional pharmaceutical practice is employed to provide suitable formulations or compositions to administer such compositions to patients. Oral administration is preferred, but any other appropriate route of administration may be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, or aerosol administration. Therapeutic formulations may be in the form of liquid solutions or suspensions (as, for example, for intravenous administration) for oral administration, formulations may be in the form of liquids, tablets, or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are described, for example, in "Remington's Pharmaceutical Sciences," Mack Publishing Company, Easton, Pa. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes.

65 If desired, slow release or extended release delivery systems may be utilized. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or

polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate, and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

In general, cytosine-containing or cytidine-containing compounds, such as CDP-choline, are administered at a dosage appropriate to the effect to be achieved and are typically administered in unit dosage form. As noted above, 15 the preferred route of administration for most indications is oral.

An effective quantity of a cytidine-containing or cytosine-containing compound is employed to treat the conditions described herein. The exact dosage of the compound may be dependent, for example, upon the age and weight of the recipient, the route of administration, and the severity and nature of the symptoms to be treated. In general, the dosage selected should be sufficient to prevent, ameliorate, or treat the condition, or one or more symptoms thereof, without producing significant toxic or undesirable side effects.

In the case of CDP-choline, there have been no reported cases of overdoses. CDP-choline toxicity is largely self-limiting, ingestion of large amounts in preclinical studies 30 shows common cholinergic symptoms (salivation, lacrimation, urination, defecation, and vomiting).

#### Other Embodiments

Preferably, cytidine-containing and cytosine-containing compounds as described herein are used for the treatment of human patients, but may also be used to treat any other mammal, for example, any pet or domesticated livestock. Any cognitive or behavioral problems associated with the types of altered brain chemistry described herein may be improved with cytidine-containing or cytosine-containing compounds, such as CDP-choline.

In addition, normal brain chemistry may also be enhanced by the administration of cytidine-containing or cytosine-containing compounds, with improvements in cognitive performance being the result.

For any of these additional uses, the cytidine-containing or cytosine-containing compound is administered by the general methods described herein.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the 65 essential features hereinbefore set forth, and follows in the scope of the appended claims.

Other embodiments are within the claims.

What is claimed is:

1. A method of preventing or ameliorating a stimulant-induced disorder in a subject exposed to a stimulant, said method comprising administering to said subject a therapeutically-effective amount of a cytidine-containing compound, cytosine-containing compound, or a combination thereof.
2. The method of claim 1 wherein an active ingredient in said cytidine-containing compound comprises CMP, CDP, CTP, dCMP, dCDP, dCTP, or a combination thereof.
3. The method of claim 1, wherein said stimulant is cocaine.
4. The method of claim 1, wherein said subject is human.
5. A method of preventing or ameliorating stimulant-induced cerebral vasoconstriction sequelae in a mammal, said method comprising administering to said mammal a therapeutically-effective amount of a cytosine-containing compound, cytidine-containing compound, or a combination thereof.
6. The method of claim 5, wherein said cerebral vasoconstriction sequelae comprises cerebral ischemia, a neuropathology, a neurological deficit, altered brain chemistry, a reduced level of task mastering, cognitive impairment, a behavioral change, a vegetative response, mental deterioration, altered conditioned avoidance or auditory response parameter, motor activity impairment, or a combination thereof.
7. The method of claim 5, wherein said cerebral vasoconstriction sequelae is characterized by symptoms resulting from an altered concentration or activity of a phosphomonoester, phosphodiester, phosphocreatine, nucleotide, triphosphate, inorganic phosphorus, Pcr/Pi ratio,  $\beta$ -NTP/Pcr ratio, cerebral phosphorus metabolite, phospholipid precursor, cellular or organelle phospholipid synthesis, membrane synthesis, tyrosine hydroxylase activity, dopamine or dopamine metabolism, bioenergetic function, fatty acid release, neutral acid, phosphatidylcholine or glycerophospholipid degradation, glucose, pyruvate, acetylcholine, norepinephrine, vasodilation, synaptosomal phosphorylation, mitochondrial ATPase or  $\text{Na}^+ - \text{K}^+$  ATPase sensitivity, phospholipase A2 activation, EEG parameter, cardiovascular or respiratory parameter, or a combination thereof.
8. The method of claim 5, wherein said mammal is a human.
9. The method of claim 8, wherein said human is a pregnant woman or a child with antenatal exposure to a stimulant.
10. The method of claim 8, wherein said human has a stimulant craving.
11. The method of claim 8, wherein said human has a stimulant dependency.
12. The method of claim of claim 5, wherein said cerebral vasoconstriction results from exposure to a substance that causes cerebral vasoconstriction.
13. The method of claim 12, wherein said substance is cocaine.
14. The method of claim 5, wherein said cytidine-containing compound further comprises choline.
15. The method of claim 14, wherein said cytidine-containing compound is CDP-choline.
16. The method of claim 5, wherein said cytidine-containing compound is CDP.

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. : 6,103,703  
DATED : August 15, 2000  
INVENTOR(S) : Perry F. Renshaw

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.

Replace title "CYTIDINE-CONTAINING AND CYTOSINE-CONTAINING COMPOUNDS AS TREATMENTS FOR STIMULANT EXPOSURE" with  
-- USE OF CYTIDINE-CONTAINING AND CYTOSINE-CONTAINING COMPOUNDS AS TREATMENTS FOR STIMULANT EXPOSURE --;

Column 1.

Line 15, replace "widespread:" with -- widespread. --.

Signed and Sealed this

Twenty-first Day of May, 2002

*Attest:*



Attesting Officer

JAMES E. ROGAN  
Director of the United States Patent and Trademark Office

J. Satoh et al. (*Euro. J. Pharmacol.* 351:155-162 (1998) – entered December 6, 2004



## Involvement of adenosine A<sub>2A</sub> receptor in sleep promotion

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### Abstract

We examined the sleep-modulatory effects of four adenosine agonists, namely, (1) 2-(4-(2-carboxyethyl)phenyl)ethylamine-adenosine-5'-N-ethylcarboxamidoadenosine (CGS21680), a highly selective adenosine A<sub>2A</sub> receptor agonist; (2) 2-(4-(2-aminocarbonylcarboxyethyl)phenyl)ethylamine-adenosine-5'-N-ethylcarboxamidoadenosine (APEC), a selective adenosine A<sub>2A</sub> receptor agonist; (3) 5'-N-ethyl-carboxamidoadenosine (NECA), a nonselective adenosine A<sub>1</sub>/A<sub>2</sub> receptor agonist; and (4) N<sup>6</sup>-cyclopentyladenosine (CPA), a selective adenosine A<sub>1</sub> receptor agonist. Each agonist was administered in the subarachnoid space underlying the rostral basal forebrain of rats through chronically implanted cannulae at the rate of 0.02, 0.2, 2.0, 12.0, or 20.0 pmol/min over a 6-h period starting from 2300 h, which period is the active phase of the animals. CGS21680, APEC, and NECA produced significant increases in the total amounts of non-rapid-eye-movement (NREM) sleep and rapid-eye-movement (REM) sleep after at least one dose within the range of administration rates. CPA did not produce any significant increase in the total amount of either type of sleep at any of the above administration rates, but instead suppressed REM sleep at the administration rates of 12.0 and 20.0 pmol/min. These results indicate that the activities of adenosine A<sub>2A</sub> receptors are crucially involved in the promotion of sleep. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Adenosine A<sub>2A</sub> receptor; Non-rapid-eye-movement (NREM) sleep; Rapid-eye-movement (REM) sleep; Brain temperature; (Rat)

### 1. Introduction

A series of papers from this and other laboratories during the past 15 yr showed prostaglandin D<sub>2</sub> to induce physiological sleep in mice, rats, monkeys, and probably in humans (Hayaishi, 1997). The site of action of prostaglandin D<sub>2</sub> for the promotion of sleep was located on the ventral surface of the rostral basal forebrain in rats, and administration of prostaglandin D<sub>2</sub> in any other part of the brain was hardly effective (Matsumura et al., 1994). More recently, we found that CGS21680, a selective adenosine A<sub>2A</sub> receptor agonist, also promoted sleep when it was infused into or near the prostaglandin D<sub>2</sub>-sensitive zone, namely, the subarachnoid space underlying the rostral basal forebrain. The sleep profile induced by CGS21680 was clearly different from that induced by N<sup>6</sup>-cyclohexyladenosine (CHA), a selective adenosine A<sub>1</sub> receptor agonist. The sleep-promoting effect of the former was

attenuated by pretreatment with (S)-1,3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl)xanthine (KF17837), a selective adenosine A<sub>2</sub> receptor antagonist. In the light of these results, we suggested that the activation of adenosine A<sub>2A</sub> receptors was involved in the promotion of sleep (Satoh et al., 1996).

Several lines of experimental evidence indicate that adenosine is involved in the regulation of sleep-wake phenomena (Hajnal et al., 1973; Radulovacki et al., 1983a; Rainnie et al., 1994; Portas et al., 1997). To date, the receptors for adenosine have been pharmacologically and structurally classified into A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> (reviewed by Fredholm et al., 1994). A role for the adenosine A<sub>1</sub> receptor in the promotion of sleep has been proposed, based on the findings that the intraperitoneal injection of approximately 0.3–9 μmol/kg of N<sup>6</sup>-(L-phenylisopropyl)adenosine (L-PIA), CHA, or CPA, all selective adenosine A<sub>1</sub> receptor agonists, and the direct administration of approximately 0.3–30 nmol of CPA into the lateral ventricle promoted deep slow wave sleep in rats (Radulovacki et al., 1982, 1983b; Ticho and Radulovacki, 1991; Benington et al., 1995; Schwierin et al., 1995). On

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the other hand, the intraperitoneal injection of selective adenosine  $A_{2A}$  receptor agonists, such as CGS21680 (5.6–560 nmol/kg) and 2-hexynyl-5'-N-ethylcarboxamide adenosine (2HE-NECA; 77–770 nmol/kg) did not promote sleep in rats (Monti et al., 1995; Bertorelli et al., 1996).

In order to further clarify the involvement of adenosine  $A_{2A}$  receptors in sleep promotion, we compared the sleep-modulatory potencies of four adenosine agonists that have different spectra of affinity and selectivity for the adenosine receptor subtypes. We now confirmed and extended results of our previous study on the effect of CGS21680. We also examined the effects of three other adenosine agonists, namely, APEC, NECA, and CPA. The  $K_1$  (nM) values of these agonists for adenosine  $A_1$ ,  $A_{2A}$ , and  $A_3$  receptors and the  $EC_{50}$  (nM) values of these agonists for the adenosine  $A_{2B}$  receptor listed in Table 1 (Hide et al., 1991; Röviks and Reppert, 1992; Kim et al., 1994; Daly and Jacobson, 1995). CGS21680 is a highly selective adenosine  $A_{2A}$  receptor agonist, whereas APEC is also a selective adenosine  $A_{2A}$  receptor agonist, but with less selectivity for this receptor subtype than CGS21680. NECA is a nonselective adenosine  $A_1/A_2$  receptor agonist. In contrast, CPA is a selective adenosine  $A_1$  receptor agonist. All these compounds are water soluble, which property is essential for our sleep research that involves a continuous infusion method, since a solvent like dimethyl sulfoxide (DMSO) itself often changes the sleep–wake pattern (personal observation).

As the sleep-promoting effect of CGS21680 was initially found in a nocturnal experiment (Saoh et al., 1996), we also examined the sleep-modulatory effect of CGS21680 by administering it over a 6-h period during the rest phase of the animal, i.e., the counter-period of the nocturnal experimental period, to determine whether the effect of the compound is influenced by the timing of its administration.

The results of the present study together with those of our previous study clearly indicate that the adenosine  $A_{2A}$  receptor is involved in sleep promotion. This effect of adenosine  $A_{2A}$  receptor agonist is observed during the night but not during the day.

Table 1  
Receptor selectivity of the adenosine agonists

Agonist	Class	$K_1$ (nM)			$EC_{50}$ (nM)
		$A_1$	$A_{2A}$	$A_3$	
CGS21680	$A_{2A}$	2600 <sup>a</sup>	15 <sup>a</sup>	584 <sup>ad</sup>	needy inactive <sup>b</sup>
APEC	$A_{2A}$	400 <sup>a</sup>	5.7 <sup>a</sup>	50 <sup>a</sup>	needy inactive <sup>b</sup>
NECA	$A_1/A_2$	5.3 <sup>a</sup>	10 <sup>a</sup>	110 <sup>a</sup>	1900 <sup>b</sup>
CPA	$A_1$	0.6 <sup>ad</sup>	462 <sup>ad</sup>	240 <sup>ad</sup>	66000 <sup>b</sup>

<sup>a</sup>Taken from Kim et al. (1994).

<sup>b</sup>Taken from Röviks and Reppert (1992).

<sup>c</sup>Taken from Hide et al. (1991).

<sup>d</sup>Taken from Jacobson et al. (1993).

## 2. Materials and methods

### 2.1. Animals

Adult male rats of the Sprague–Dawley strain, weighing 300–350 g, were supplied from Japan SLC ( $n = 162$ ). They were maintained for 7–10 days before the surgical operation under constant environmental conditions of controlled relative humidity ( $60 \pm 5\%$ ) and room temperature ( $25 \pm 0.5^\circ\text{C}$ ) on a 12-h light/12-h dark schedule (lights on at 0800 h), which were the same environmental conditions as those of the experimental chambers. They were permitted free access to food and water.

### 2.2. Surgery

Under deep pentobarbital anesthesia (50 mg/kg body weight, i.p.), each rat underwent a surgical operation for the implantation of electrodes to record the electroencephalogram (EEG) and electromyogram (EMG) and of a thermistor probe to monitor brain temperature. In some cases, implantations of electrodes to record the electrooculogram (EOG) were performed in addition. For delivery of agonists, paired stainless-steel cannulae were inserted according to the stereotaxic coordinates from the brain atlas of Paxinos and Watson (1986): AP + 1.5 mm, ML + 1.7 mm, D – 8.3 mm from the brain surface with an angle of 10° towards the midline from the parasagittal plane. Thus, the tips of the cannulae were situated in the subarachnoid space underlying the rostral basal forebrain.

### 2.3. Experimental protocol

The protocol adopted in this study was essentially the same as that described before (Matsumura et al., 1994; Saoh et al., 1996). After a period of 7–10 days for recovery from surgery, the rats were set inside the experimental chamber, the interior of which was maintained under the same conditions as described above. Continuous infusion of saline through the infusion cannulae was commenced, and this infusion lasted until the end of the experimental session except for the period when the test compound was infused. The infusion speed of vehicle and test solution was set at 0.2  $\mu\text{l}/\text{min}$ . After a period of 4 days for acclimation to the experimental milieu, continuous recordings of EEG, EMG and brain temperature were started at 2000 or 0800 h. In some cases, continuous recordings of EOG were also additionally performed. The subsequent two 24-h periods were designated as baseline day and experimental day, respectively. Each rat received only one experimental infusion according to the following protocols:

CGS21680, APEC, NECA and CPA were each dissolved in saline to make 0.05, 0.5, 3.0, 30 and 30  $\mu\text{M}$  solutions. The bilateral infusion of each test solution re-

sulted in the total administration rate of 0.02, 0.2, 2.0, 12.0 and 20.0 pmol/min, respectively. The baseline day was started at 2000 h. The continuous infusion of saline was replaced by that of a particular test solution from 2300 h to 0500 h on the experimental day. For the diurnal infusion experiments, CGS21680 was dissolved in the saline solution to make 0.5, 5.0 and 30  $\mu$ M solutions (corresponding to 0.2, 2.0, and 12.0 pmol/min, respectively, for the total rate of the bilateral infusion). The baseline day was started at 0800 h. The continuous infusion of saline was replaced by that of the CGS21680 solution between 1100 and 1700 h on the experimental day.

After the end of each experimental session, 2% methylene blue solution was continuously administered for 3 h at the infusion rate of 0.2  $\mu$ l/min. Then, the rats were deeply anesthetized with pentobarbital sodium and perfused transcardially with saline solution, followed by 0.1 M phosphate buffer/4% paraformaldehyde. The location of cannula tips was identified from blue spots on the ventral surface of rostral basal forebrain. In some cases, the brains were postfixed and then transferred to 0.1 M phosphate buffer/20% sucrose until equilibration. The brains were cut coronally at a thickness of 50  $\mu$ m with a freezing microtome. The location of cannula tips was also identified from the cannula traces within the section.

#### 2.4. Analytical procedure

The recordings of EEG and EMG from each animal were scored as; non-rapid-eye-movement (NREM) sleep, rapid-eye-movement (REM) sleep, or wakefulness by visual inspection according to the criteria described earlier (Matsumura et al., 1994). Thus, light slow wave sleep and deep slow wave sleep were combined and treated as NREM sleep. An episode of vigilance state lasting less than 15 s was not treated as an independent episode but was included in the preceding episode. The values obtained from experimental administration were compared with corresponding values from the baseline recordings (control), and statistical analysis was done with a paired *t*-test. The changes in sleep periods induced by different doses of a given adenosine receptor agonist, were compared by means of one-way analysis of variance (One-way ANOVA). *P* values were corrected for multiple comparisons according to Scheffé's *F* post-hoc procedure. To compare the changes in sleep periods induced by CGS21680 with those induced by the other adenosine receptor agonists at the respective infusion rates, we used an One-way ANOVA. *P* values were corrected according to Dunnett's post-hoc procedure for comparing a control to all other means. *P* < 0.05 was considered significant.

#### 2.5. Chemicals

Agonists 2-(4-(2-carboxyethyl)phenylethylamino)-adenosine-5'-N-ethylcarboxamidoadenosine (CGS21680),

5'-N-ethylcarboxamidoadenosine (NECA), and *N*<sup>6</sup>-cyclopentyladenosine (CPA) were purchased from Research Biochemicals (Natick, MA). The agonist, 2-(4-(2-aminoethylaminocarbonyl)ethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine (APEC), was provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health. (Contract N01MH3003)

### 3. Results

#### 3.1. Dose-response studies

The means and standard error values for the total amounts of NREM sleep and REM sleep that occurred during the 6-h control periods (open circles and black bars) and experimental periods (closed circles and black bars) are summarized in Fig. 1 for the four adenosine receptor agonists given at different infusion rates. During the 6-h control period, the rats of each group showed nearly identical periods of sleep, about 100 min for NREM sleep and about 15 min for REM sleep.

Firstly, we evaluated the changes in sleep that occurred at different infusion rates of an adenosine receptor agonist. ANOVA revealed a significant variance in NREM sleep (CGS21680: *F*(4,34) = 23.69, *P* < 0.01; APEC: *F*(4,25) = 19.51, *P* < 0.01; NECA: *F*(3,24) = 4.65, *P* < 0.03; CPA: *F*(4,29) = 3.24, *P* < 0.05) and in REM sleep (CGS21680: *F*(4,34) = 6.10, *P* < 0.01; APEC: *F*(4,25) = 12.11, *P* < 0.01; NECA: *F*(3,24) = 9.73, *P* < 0.01; CPA: *F*(4,29) = 3.99, *P* < 0.05).

CGS21680 and APEC, selective adenosine  $A_{2A}$  receptor agonists, showed dose-dependent increases in NREM sleep within the dose range of 0.02–2.0 pmol/min. The increases in NREM sleep that occurred at the infusion rate of 2.0 pmol/min from the respective baseline values were  $97.5 \pm 4.4$  min ( $a 99.5 \pm 6.9\%$  increase; paired *t*-test, *P* < 0.001) for CGS21680 and  $116.4 \pm 8.3$  min ( $a 121.8 \pm 14.3\%$  increase; paired *t*-test, *P* < 0.001) for APEC, amounts which were significantly larger than those recorded at the infusion rate of 0.2 pmol/min (Scheffé's *F*, *P* < 0.01). The NREM sleep-promoting effect of CGS21680 reached its plateau at the administration rate of 2.0 pmol/min and was not further modified at the rate of 12.0 or 20.0 pmol/min. The NREM sleep-promoting effect of APEC was attenuated in a dose-dependent manner above 2.0 pmol/min, and this attenuation reached statistical significance at the infusion rate of 20.0 pmol/min (Scheffé's *F*, *P* < 0.01).

NECA, a nonselective  $A_1/A_2$  adenosine receptor agonist, also produced an increasing tendency to NREM sleep within the dose range of 0.02–2.0 pmol/min. The increase in NREM sleep from the baseline value at the infusion rate of 2.0 pmol/min was  $67.1 \pm 16.3$  min ( $a 74.5 \pm 17.9\%$  increase; paired *t*-test, *P* < 0.001); however, this amount

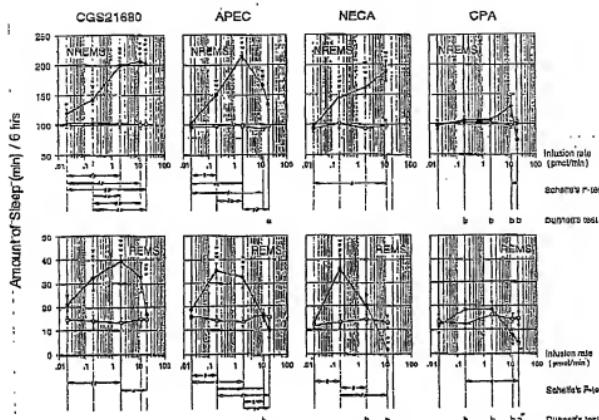


Fig. 1. Effects of adenosine agonists on sleep. CGS21680, APEC, NECA, and CPA were separately and continuously infused into the subcutaneous space underlying the rostral basal forebrain over a 6-h period during the active phase of rats, at administration rates of 0.02, 0.2, 2.0, 12.0, or 20.0 pmol/min. The total amounts (mean  $\pm$  S.E.M.) of NREM sleep and REM sleep during the 6-h control period (open circles and black bars) or the experimental period (closed circles and black bars) at the respective administration rates are shown.  $n = 5-11$  for each group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , vs. control by paired  $t$ -test. The changes in sleep induced by an adenosine agonist given at different infusion rates were compared with each other. Significance of differences was determined by one-way analysis of variance (One-way ANOVA) followed by Scheffé's  $F$ -test. Bars with arrowheads on both sides show the significance of differences (\*, \*  $P < 0.05$ , \*\*, \*\*  $P < 0.01$ ) between the changes in sleep at the infusion rates indicated. The changes in sleep induced by CGS21680 at the various infusion rates were compared with those induced by APEC, NECA, and CPA at the respective rates. Significance of difference—(a)  $P < 0.05$ ; (b)  $P < 0.01$ —was determined by One-way ANOVA followed by Dunnett's test.

was not significantly different from that found at the infusion rate of 0.2 pmol/min (Scheffé's  $F$ ,  $P = 0.745$ ). At the infusion rate of 12.0 pmol/min, NECA produced unusual synchronization of the EEG. NREM sleep episodes with a duration longer than 600 s often appeared in the latter half of the administration period, whereas the normal duration of an NREM sleep episode of a rat was within 450 s under our experimental conditions (data not shown).

As for REM sleep, CGS21680 and APEC showed a bell-shaped, dose-response relationship. The most effective dosage for the respective agonists and the increase in response to it were as follows: 2.0 pmol/min,  $26.3 \pm 1.4$  min (a  $305 \pm 77.0\%$  increase; paired  $t$ -test,  $P < 0.001$ ) for CGS21680; 0.2 pmol/min,  $21.3 \pm 3.3$  min for APEC (a  $177.3 \pm 32.8\%$  increase; paired  $t$ -test,  $P < 0.001$ ). These increases were significantly larger than those found at the infusion rate of 0.02 (Scheffé's  $F$ ;  $P < 0.01$  for CGS21680,  $P < 0.05$  for APEC) and 20.0 pmol/min (Scheffé's  $F$ ;  $P < 0.01$  for CGS21680 and APEC).

NECA also showed this bell-shaped, dose-response relationship. The greatest increase appeared at the infusion rate of 0.2 pmol/min, which was  $22.5 \pm 3.1$  min (a  $206.7 \pm 40.1\%$  increase; paired  $t$ -test,  $P < 0.001$ ). This increase

was significantly greater than increases at the infusion rates of 0.02, 2.0 (Scheffé's  $F$ ,  $P < 0.05$ ), and 12.0 pmol/min (Scheffé's  $F$ ,  $P < 0.01$ ).

In contrast to these agonists, CPA, a selective adenosine A<sub>1</sub> receptor agonist, caused negligible changes in the amount of NREM sleep or REM sleep within the dose range of 0.02–2.0 pmol/min. Further increases in dosage did not produce a consistent response in NREM sleep, but did cause a significant decrease in REM sleep from the baseline values (paired  $t$ -test,  $P < 0.05$ ).

Secondly, we compared the changes in sleep induced by the four adenosine agonists at the respective infusion rates. ANOVA revealed a significant variance in NREM sleep (0.2 pmol/min:  $F(3,23) = 7.34$ ,  $P < 0.01$ ; 2.0 pmol/min:  $F(3,31) = 17.67$ ,  $P < 0.01$ ; 12.0 pmol/min:  $F(3,23) = 5.30$ ,  $P < 0.01$ ; 20.0 pmol/min:  $F(2,15) = 15.41$ ,  $P < 0.01$ ) and in REM sleep (0.2 pmol/min:  $F(3,23) = 3.69$ ,  $P < 0.05$  pmol/min; 2.0 pmol/min:  $F(3,31) = 11.52$ ,  $P < 0.01$ ; 12.0 pmol/min:  $F(3,23) = 12.52$ ,  $P < 0.01$ ; 20.0 pmol/min:  $F(2,15) = 3.87$ ,  $P < 0.05$ ).

The changes in NREM sleep induced by CGS21680 were significantly different from those induced by CPA at the infusion rates of 0.2, 2.0, 12.0 and 20.0 pmol/min

(Dunnett two-tailed,  $P < 0.01$ ), and the changes induced by the former were also significantly different from that induced by APEC at the infusion rate of 20.0 pmol/min (Dunnett two-tailed,  $P < 0.05$ ). The changes in REM sleep induced by CGS21680 were significantly different from those caused by CPA at the infusion rates of 0.2, 2.0, 12.0 and 20.0 pmol/min (Dunnett two-tailed,  $P < 0.01$  for 0.2, 2.0, 12.0 pmol/min,  $P < 0.05$  for 20.0 pmol/min), and from those caused by NECA at the infusion rate of 2.0 and 12.0 pmol/min (Dunnett two-tailed,  $P < 0.01$ ). At the infusion rate of 12.0 pmol/min, a significant difference was also seen in the changes between CGS21680 and APEC (Dunnett two-tailed,  $P < 0.01$ ).

### 3.2. Profiles of the changes in sleep and brain temperature induced by the adenosine agonists

The 24-h profiles of sleep and of brain temperature at the administration rate of 2.0 pmol/min of the adenosine agonists are shown in Fig. 2A and B, respectively. At this administration rate, CGS21680 produced increases in NREM sleep and REM sleep, both with a maximal magnitude.

CGS21680 and APEC produced the increases in NREM sleep from the first hour of their administration, and increases in REM sleep from the second or third hour. These increases in NREM sleep and REM sleep lasted throughout the administration period. In the case of NECA,

a lag period of 2 h preceded the beginning of the increase in NREM sleep, and the increase in REM sleep occurred during the last 2 h of the administration period. In contrast, CPA appeared to cause a biphasic response composed of an initial decrease and a subsequent increase in the profile of NREM sleep, whereas it caused solely an increase in REM sleep during the last 2 h of the administration period. A further increase in the dose of CPA also produced a biphasic response in NREM sleep, but a continuous decrease in REM sleep from the first hour of its administration (data not shown).

CGS21680 and APEC caused a gradual decrease of the profile of brain temperature from the second hour of the administration period, which decrease lasted during the remainder of the infusion period. NECA also caused a drop in temperature but only after a lag period of 4 h. CPA caused only marginal changes in the brain temperature except for the fifth hour of the administration period, at which time the temperature decreased slightly.

### 3.3. Effect of diurnal treatment with CGS21680 on sleep

Diurnal infusion of CGS21680 also caused a dose-dependent increasing tendency in NREM sleep, but a dose-dependent decreasing tendency in REM sleep (Fig. 3). At the infusion rate of 0.2, 2.0 and 12.0 pmol/min, the increases in NREM sleep from their respective baseline values were  $6.7 \pm 3.1$  min (paired *t*-test, n.s.),  $15.4 \pm 5.4$

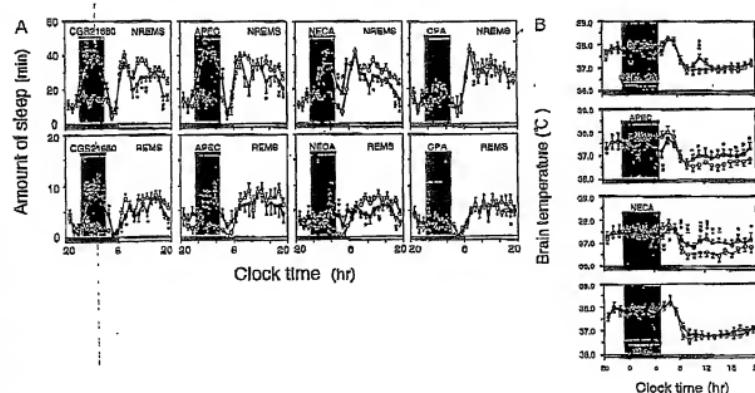


Fig. 2. Twenty-four-hour profiles of sleep (A) and brain temperature (B) on the control day (open circles) and the experimental day (closed circles). CGS21680, APEC, NECA, and CPA were separately administered into the subarachnoid space underlying the rostral basal forebrain bilaterally at the total infusion rate of 2.0 pmol/min during the middle part (2300 h–0500 h) of the active period of the animals.  $n = 7$ –11 per group; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , vs. control by paired *t*-test.

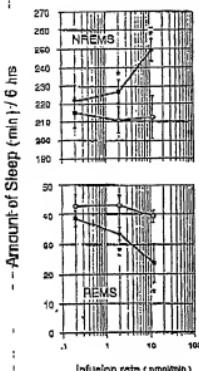


Fig. 3. Effect of bilateral administration of CGS21680 on sleep. CGS21680 was continuously infused into the subarachnoid space underlying the rostral basal forebrain bilaterally at the total infusion rate of 0.2, 2.0, or 12 pmol/min over a 6-h period (1100 h–1700 h) during the rest period of the rat. The total amounts (mean  $\pm$  S.E.M.) of NREM sleep and REM sleep during the 6-h control (open circles) and black bars) and experimental (closed circles and black bars) periods are shown.  $n = 7$ –13 per each group; \* $P < 0.05$ ; \*\* $P < 0.01$ , vs. control by paired  $t$ -test.

min (paired  $t$ -test,  $P < 0.05$ ), and  $33.2 \pm 5.7$  min (paired  $t$ -test,  $P < 0.01$ ), respectively; whereas the decreases in REM sleep were  $4.2 \pm 2.5$  min (paired  $t$ -test, n.s.),  $9.8 \pm 2.7$  min (paired  $t$ -test,  $P < 0.01$ ) and  $15.7 \pm 6.3$  min (paired  $t$ -test,  $P < 0.05$ ), respectively. ANOVA revealed a significant variance among the changes in NREM sleep ( $F(2,16) = 3.65$ ,  $P < 0.05$ ), but not in REM sleep ( $F(2,16) = 1.21$ ,  $P = 0.32$ ). However, no significant difference was found between any pairs of the changes in NREM sleep as evaluated in Scheffé's  $F$ -test.

#### 4. Discussion

The data from the present study, demonstrating that adenosine  $A_{2A}$  receptor agonists promoted sleep when administered directly into the rostral basal forebrain during the night, i.e., the active phase of the rats, are consistent with our previous findings. These data further support our hypothesis that the activity of adenosine  $A_{2A}$  receptors is involved in the promotion of sleep.

##### 4.1. Administration of adenosine agonists to ventral and rostral part of forebrain

The differences between the marked increases in NREM sleep and REM sleep induced by CGS21680, and the

negligible changes in sleep induced by CPA clearly indicate that activation of adenosine  $A_{2A}$  receptors is involved in the promotion of sleep. In addition, the increases in NREM sleep and REM sleep induced by APEC and NECA at the relatively low dosages appeared to be due to the activity of adenosine  $A_{2A}$  receptors. Attenuation of sleep-promoting potency at relatively high dosages of APEC, as well as the lag period before the start of the increases in sleep and unusual pattern of EEG during the treatment with NECA, might be explained by the additional activation of adenosine  $A_1$  and  $A_3$  receptors. The activation of adenosine  $A_1$  receptors in particular may have produced initially a subtracting and, subsequently, an additive effect on the  $A_{2A}$ -mediated increases in NREM sleep, since CPA, an adenosine  $A_1$  receptor agonist, produced a biphasic response composed of an initial decrease and a subsequent increase in NREM sleep when it was administered in the subarachnoid space of the rostral basal forebrain in this study. Activation of adenosine  $A_3$  receptors might suppress sleep, because it was reported that histamine, which promotes wakefulness, is released from mast cells in response to the administration of 2-chloro-N<sup>6</sup>-(3-jodobenzyl)adenosine-5'-N-methylcarboxamide (2-Cl-IB-MECA), a selective adenosine  $A_3$  receptor agonist (Van Schaick et al., 1996).

Recently, it was reported that 8-(3-chlorostyryl)caffiene (CSC) and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-c]-1,2,4,4-triazolo-[1,5-c]-pyrimidine (SCH5261), both selective adenosine  $A_{2A}$  receptor antagonists, increase locomotion activity and, thus, wakefulness (Bertorelli et al., 1996; Jacobson et al., 1999). These reports suggest that a certain part of physiological sleep is produced through the activity of adenosine  $A_{2A}$  receptors.

On the other hand, CPA, an adenosine  $A_1$  receptor agonist, did not produce a consistent increase in NREM sleep and, instead, suppressed REM sleep in the present study. These effects were similar to those of CHA on sleep that were found in our previous study and to the results obtained following the intraperitoneal injection of CPA (1.0 mg/kg body weight) in rats (Schwierin et al., 1996). However, it was reported that the intraperitoneal and intracerebroventricular administration of CPA, and the microinjection of CPA into preoptic area increase the slow-wave activity in NREM sleep (Ticho and Radulovacki, 1991; Benington et al., 1995; Schwierin et al., 1996). Since we did not carry out a spectral analysis of EEG, it is difficult to know whether our treatment with CPA increased the slow-wave activity in NREM sleep or not. Furthermore, it is also unclear whether our experimental site was mainly responsible for the adenosine  $A_1$  receptor-mediated promotion of slow-wave activity in NREM sleep or not.

##### 4.2. Effects of adenosine agonists on brain temperature

It is well known that brain temperature decreases during the natural sleep period (reviewed by McGinty and Szymu-

sia, 1990). Similar findings were obtained in the present study. The decrease in the brain temperature induced by CGS21680, APEC, and NECA started along with or after the beginning of the increase in NREM sleep, and did not precede the change in NREM sleep. In these cases, the magnitude of the changes in brain temperature remained within the range of natural circadian variation. The continuous infusion of CPA produced a small decrease in brain temperature only at the fifth hour of administration, around which period a small increase in NREM sleep appeared. Other groups also showed that the microinjection of CPA (0.5 nmol) or NECA (1.0 nmol) into the preoptic area produced an approximately 0.5–1.0°C reduction in brain temperature and an increase in deep slow wave sleep (Ticha and Radulovacki, 1991). Therefore, in contrast to some cytokines that produce sleep and fever (reviewed by Kruger and Majde, 1994), an adenosine receptor agonist could produce the natural relation between sleep and brain temperature if an appropriate amount was directly administered to the brain.

#### 4.3. Effect of day time treatment with CGS21680 on sleep

There were several reports indicating a difference between the response to the day time and to the night time treatment with sleep-promoting substances. Diurnal administration of sleep substances, such as delta-sleep-inducing-peptide (DSIP), prostaglandin D<sub>2</sub>, and uridine caused almost no changes in sleep parameters (Inoué et al., 1984). Furthermore, trizolam, one of the benzodiazepines, failed to show sleep-promoting efficacy in rats when injected in the middle of their rest phase (Egger et al., 1991). Thus, it is likely that the effectiveness of exogenously administered CGS21680 and other substances is affected by intrinsic circadian factors. The total sleep period during the diurnal treatment with CGS21680 was around 260 min, and was almost consistent at the various infusion rates used in this study. This phenomenon well resembles the upper limit that total sleep does not exceed in spite of prior sleep deprivation and was named the 'ceiling effect' (Mitselberger et al., 1983). Furthermore, this might be one reason why the other research groups did not observe an increase in sleep following the intraperitoneal injection of A<sub>2A</sub> adenosine agonists during the day period (Monti et al., 1995; Bertorelli et al., 1996).

#### 4.4. For further study of a sleep-generating structure located in the rostral basal forebrain

Early studies demonstrating that chemical or electrical lesioning of a particular brain region caused the loss of sleep, whereas electrical stimulation of this region produced synchronization of the EEG, indicated that some sleep-generating structure is located in the rostral part of the brain, such as the anterior hypothalamus, preoptic area, and basal forebrain (reviewed by Jones, 1994). However, the pharmacological and anatomical characteristics of this

sleep-promoting structure have not yet been clearly elucidated.

In the present study, the tip of each infusion cannula was situated in the subarachnoid space underlying the ventral and medial part of the shell of the accumbens nucleus. In this area and in its vicinity, a dense distribution of adenosine A<sub>2A</sub> receptors was demonstrated by use of ligand binding autoradiography using [<sup>3</sup>H]CGS21680 and *in situ* hybridization with antisense RNA for the adenosine A<sub>2A</sub> receptor. Adenosine A<sub>2A</sub> receptors are known to be localized in GABA ( $\gamma$ -aminobutyric acid)-ergic neurons that coexpress enkephalin (reviewed by Ongini and Fredholm, 1996). These neurons might be responsible for the sleep-promoting effect of adenosine A<sub>2A</sub> receptor agonists.

Several brain regions have been proposed to be involved in the generation of sleep. The ventral surface of the rostral basal forebrain is considered to be the site of action of prostaglandin D<sub>2</sub> for sleep-promotion in rats (Masumura et al., 1994). The cholinergic neurons of the basal forebrain and the laterodorsal tegmentum are considered to be involved in the adenosine-induced sleep in cats (Rainnie et al., 1994; Portas et al., 1997). Some neurons in the medial preoptic area respond to local warming and prolong the period of slow wave sleep (McGinty and Szymusiak, 1990). The average number of FOS-immunoreactive cells per ventrolateral preoptic area (VLPO) sector increased with increasing percent total sleep time for spontaneously behaving rats (Sherin et al., 1996). The microinjection of muscimol, a GABA<sub>A</sub> receptor agonist, into the ventrolateral region of the pericirculectal gray and adjacent tegmentum increases the period of REM sleep (Sastré et al., 1996). The cholinceptive desynchronized sleep induction zone is localized within the dorsal tegmentum of the pons (Vanni-Mercier et al., 1989; Yamamoto et al., 1990). In contrast, the posterior hypothalamus, especially the histaminergic neurons in the tuberomamillary nucleus, participates in the regulation of wakefulness (Lin et al., 1989). The putative adenosine A<sub>2A</sub> receptor mediating the sleep-promoting system would relate directly or indirectly to the activities of these structures. Particularly, the increase in REM sleep might be explained by a disinhibition of mesopontine REM-on neurons, as mentioned by Portas et al. (1997).

For clarification of this issue, it will be necessary to decide the site of action of an adenosine A<sub>2A</sub> agonist for sleep promotion. To this end, anatomical studies with tracers will be helpful. CGS21680 should serve as a useful tool for further study of the sleep-generating structures located in the rostral and ventral part of the forebrain and their relation to other established sleep-generating structures.

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abode of some other species (an oyster crab within the shell of an oyster) causing little or no inconvenience to the host. SEE ALSO commensal. [L. *inquilinus*, an inhabitant of a place that is not his own, fr. *in*, in, + *colere*, to inhabit]

**INR** Abbreviation for international normalized ratio.

**in-sa-lu-bri-ous** (in-sä-loo-bré-üs). Unwholesome; unhealthful; usually in reference to climate. [L. *in-salubris*, unwholesome]

**in-sane** (in-sän'). 1. Of unsound mind; severely mentally impaired; deranged; crazy. 2. Relating to insanity. [L. *in*- neg. + *sanus*, sane]

**in-sani-tary** (in-sän-tärë). Injurious to health, usually in reference to an unclean or contaminated environment. SYN unsanitary. [L. *in*- neg. + *sanus*, sound]

**in-san-i-ty** (in-sän-i-të). 1. An outmoded term referring to severe mental illness or psychosis. 2. In law, that degree of mental illness which negates the individual's legal responsibility or capacity. [L. *in*- neg. + *sanus*, sound]

**criminal** I., in forensic psychiatry, a term that describes the degree of mental competence and that is defined by such currently applicable legal precedents as the American Law Institute rule, Durham rule, M'Naghten rule, and the New Hampshire rule.

I. defense, in forensic psychiatry, the use in the courtroom of I. as a mitigating factor in the defense of an individual on trial for a serious criminal offense. SEE criminal I.

**in-scrip-tio** (in-skrip-të-ö). SYN inscription. [L. fr. *in-scribo*, pp. *-scriptus*, to write on]

I. **tendin'ë**, SYN tendinous intersection.

**in-scrip-tion** (in-skrip-shün). 1. The main part of a prescription; that which indicates the drugs and the quantity of each to be used in the mixture. 2. A mark, band, or line. SYN inscription. [L. *scriptio*]

**tendinous** I., SYN tendinous intersection.

**in-sec-ta** (in-sek-tä'). The insects, the largest class of the phylum Arthropoda and the largest major grouping of living things, chiefly characterized by flight, great adaptability, vast speciation in terrestrial and freshwater environments, and possession of three pairs of jointed legs and, usually, two pairs of wings. Some are parasitic; others serve as intermediate hosts for parasites, including those that cause many human diseases. Some are wingless; others, such as the Diptera, have only one pair of wings. Respiration is by tracheoles, cuticle-lined air tubes that pass air directly to the tissues. Development in higher forms is holometabolous and passes through distinctive egg, larval, pupal, and adult stages. SYN Hexapoda. [L. pl. of *insecus*, insect, fr. *in-seco*, pp. *-secutus*, to cut into]

**in-sec-tar-i-um** (in-sek-tärë-üm). Place for keeping and breeding insects for scientific purposes. [L.]

**in-sec-ti-cide** (in-sek-ti-sid'). An agent that kills insects. [insect + L. *caedē*, to kill]

**in-sec-ti-fuge** (in-sek-ti-foo'). A substance that drives off insects. [insect + L. *fugo*, to put to flight]

**in-sec-tiv-o-ra** (in-sek-tiv'-rä). An order of small, plantigrade, placental mammals that are extremely active and often highly predaceous; they feed mostly on insects and small rodents, although the jar or potomogale of Africa feeds on fish. Eight living families include the solenodons of Cuba and Haiti, tenrecs of Madagascar, hedgehog of Europe and Asia, and shrews and moles of the U.S., Africa, and Asia. [insect + L. *voro*, to devour]

**in-sec-tiv-o-rous** (in-sek-tiv'-rä-dës). Insect-eating. [insect + L. *voro*, to devour]

**in-se-cu-ri-ty** (in-së-kür'i-të). A feeling of unprotectedness and helplessness.

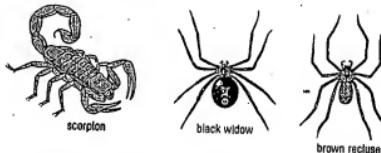
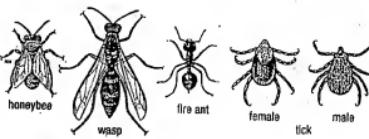
**in-semi-na-tion** (in-semi-nä-shün). Deposit of seminal fluid within the vagina, normally during coitus. SYN semination. [L. *in-semino*, pp. *-atus*, to sow or plant in; fr. *semen*, seed]

**artificial** I., the introduction of semen into the vagina other than by coitus.

**donor** I., SYN heterologous I.

**heterologous** I., artificial I. with semen from a donor who is not the woman's husband. SYN donor I.

**homologous** I., artificial I. with the husband's semen.



biting and stinging Insects and arachnids: (top) non-dangerous, (middle) potentially dangerous, (bottom) life-threatening; insects shown are not drawn to scale

**intrauterine** I. (IUI), placement of sperm that have been washed of seminal fluid directly into the uterus to bypass the cervix.

**in-se-nes-cence** (in-së-nës'ëns). The process of growing old. [L. *insenescere*, to begin to grow old]

**in-se-ni-si-ble** (in-sen'-si-bël). 1. SYN unconscious. 2. Not appreciable by the senses. [L. *in-sensibilis*, fr. *in*, neg. + *sensio*, pp. *sensus*, to feel]

**in-sert** (in'sërt). 1. An additional length of base pairs in DNA that has been introduced into that DNA. 2. An additional length of bases that has been introduced into RNA. 3. An additional length of amino acids *acyl* residues that has been introduced into a protein.

**in-ser-tion** (in-ser-shün). 1. A putting in. 2. The usually more distal attachment of a muscle to the more movable part of the skeleton, as distinguished from origin. 3. In dentistry, the intrar oral placing of a dental prosthesis. 4. Intrusion of fragments of any size from molecular to cytogenetic into the normal genome. [L. *insertio*, a planting in, fr. *insero*, *-sersus*, to plant in]

**parasol** I., SYN velamentous I.

**velamentous** I., a form of I. of the fetal blood vessels into the placenta, in which the vessels separate before reaching the placenta and develop toward it in a fold of amniotic, somewhat like the ribs of an open parasol. SYN parasol I.

**in-sheathed** (in-shéthd'). Enclosed in a sheath or capsule.

**in-sid-i-ous** (in-sid'-ëüs). Tracherous; stealthy; denoting a disease that progresses gradually with inapparent symptoms. [L. *insidiosus*, cunning, fr. *insidias* (pl.), an ambush]

**in-sight** (in'-sëth). Self-understanding as to the motives and reasons behind one's own actions or those of another's.

**in-sit** (in'-sët'). In position, not extending beyond the focus or level of origin. [L. *in*, in, + *situs*, site]

**in-so-nation** (in-sö-nä-shün). 1. Exposure to the sun's rays. 2. SYN sunstroke. [L. *insolare*, to place in the sun]

**in-sol-u-bil** (in-sö'l-bil). Not soluble.

**in-som-nia** (in-som'në-ä). Inability to sleep, in the absence of external impediments, such as noise, a bright light, etc., during the period when sleep should normally occur; may vary in degree from restlessness or disturbed slumber to a curtailment of the

normal length of sleep or to absolute wakefulness. *syn* sleeplessness. [L. *fr. in-* priv. + *somnus*, sleep]

**conditioned** *i.*, a form of insomnia resulting from conditioned behaviors that are incompatible with sleep, e.g., each time a person walks into his bedroom, his first thought is that he is not going to be able to sleep.

**subjective** *i.*, a condition characterized by the subjective experience of greatly reduced sleep, in the context of relatively normal physiologic measures of sleep.

**in-som-ni-ac** (in-som'ne-ak). 1. A sufferer from insomnia. 2. Exhibiting, tending toward, or producing insomnia.

**in-sorp-tion** (in-sôrp'shün). Movement of substances from the lumen of the gut into the blood. [L. *in*, in, + *sorbo*, to suck] *inspiration*.

**visual** *i.* with **acetic acid**, *syn* acetowhitening, cervicoscopy.

**in-sper-sion** (in-sper'shün, -zhün). Sprinkling with a fluid or a powder. [L. *insperso*, fr. *in-spergo*, pp. *-spersus*, to scatter upon, fr. *spargo*, to scatter]

**in-spi-ra-tion** (in-spi-rä'shün). *syn* inhalation (1). [L. *inspiratio*, fr. *in-spiro*, pp. *-atus*, to breathe in] *crowning* *i.*, noisy breathing associated with respiratory obstruction, usually at the larynx.

**in-spi-ra-to-ry** (in-spi-rä-tö-rë). Relating to or timed during inhalation.

**in-spir'e** (in-spir'). *syn* inhale.

**in-spi-rom-e-ter** (in-spi'rom-ë-ter). An instrument for measuring the force, frequency, or volume of inspirations. [L. *in-spiro*, to breathe in, + G. *metron*, measure]

**in-spi-sa-tion** (in-spi'shün). To perform or undergo inspiration.

**in-spi-sa-tion** (in-spi-sä'shün). 1. The act of thickening or condensing, as by evaporation or absorption of fluid. 2. An increased thickening or diminished fluidity. [L. *in*, intensive, + *spisko*, pp. *-atus*, to thicken].

**in-spi-sa-tor** (in-spi-sä'tör). An apparatus for evaporating fluids. *in-sta-bil-i-ty* (in-stä-bil'i-të). The state of being unstable, or lacking stability.

**detrusor** *i.*, involuntary detrusor contractions that may occur at bladder volumes below capacity; *syn* detrusor hyperreflexia.

**spinal** *i.*, the inability of the spinal column, under physiologic loads, to maintain its normal configuration; may result in damage to the spinal cord or nerve roots or lead to the development of a painful spinal deformity.

**in-star** (in'shär). Any of the successive nymphal stages in the metamorphosis of hemimetabolous insects (simple or incomplete metamorphosis), or the stages of larval change by successive molts that characterize the holometabolous insects (complex or complete metamorphosis). [L. *form*]

**in-step**. The arch, or highest part of the dorsum of the foot, *SEE ALSO* tarsus.

**in-stil-la-tion** (in-sti-lä'shün). Dropping of a liquid on or into a body part. [L. *instillatio*, fr. *in-stillo*, pp. *-atus*, to pour in by drops, fr. *stilla*, a drop]

**in-stil-la-to-ry** (in-sti-lä-tö-ri). A device for performing instillation. *syn* dropper.

**in-stinct** (in'stinkt). 1. An enduring disposition or tendency of an organism to act in an organized and biologically adaptive manner characteristic of its species. 2. The unreasoning impulse to perform some purposive action without an immediate consciousness of the end to which that action may lead. 3. In psychoanalytic theory, the forces or drives assumed to exist behind the tension caused by the needs of the id. [L. *instinctus*, impulse]

**aggressive** *i.*, *syn* death *i.*

**death** *i.*, an *i.* of living creatures toward self-destruction, death, or a return to the inorganic lifelessness from which they arose. *syn* aggressive *i.*

**ego** *i.*'s, self-preservation needs and self-love, as opposed to object love; drives that are primarily erotic.

**herd** *i.*, tendency or inclination to band together with and share the customs of others of a group, and to conform to the opinions and adopt the views of the group. *syn* social *i.*

**life** *i.*, the *i.* of self-preservation and sexual procreation; the basic urge toward preservation of the species. *syn* sexual *i.*

**sexual** *i.*, *syn* herd *i.*

**social** *i.*, *syn* herd *i.*

**in-stin-cive**, **in-stinc-tu-al** (in-stink'tiv, -stink'choo-äl). Relating to instinct.

**in-stru-ment** (in'stroo-men't). A tool or implement. [L. *instrumentum*]

**diamond cutting** *i.*'s, in dentistry, cylinders, disks, and other cutting *i.*'s to which numerous small diamond pyramids have been attached by a plating of metal.

**hearing** *i.*, *syn* hearing aid.

**Krueger** *i.* stop, a mechanical device limiting the insertion of a root canal *i.* into a canal.

**plugging** *i.*, *syn* plugger.

**purse-string** *i.*, an intestinal clamp with jaws at an angle to the handle; when closed across the bowel, large grooved interdigitating serrations allow passage of a straight needle and suture through each side to form a purse-string suture, after which the clamp is removed.

**Sabouraud-Noiré** *i.*, an obsolete device for measuring the quantity of x-rays by means of the change in color of a disk of barium platinocyanide which exposure to them produces; the unit used in this method is called *i.* *SEE* erythema dose.

**stereotactic** *i.*, *stereotaxic *i.*, an apparatus attached to the head, used to localize precisely an area in the brain by means of coordinates related to intracerebral structures.*

**test handle** *i.*, a root canal *i.* the handle of which is similar to a collet chuck and which can be secured in position on the root canal *i.* to adjust its effective length.

**in-stru-men-tar-i-um** (in'stroo-men-tär'ë-üm). A collection of instruments and other equipment for an operation or for a medical procedure.

**in-stru-men-ta-tion** (in'stroo-men-täshün). 1. The use of instruments. 2. In dentistry, the application of armamentarium in a restorative procedure.

**in-suc-ca-tion** (in'süksä'shün). Maceration or soaking, especially of a crude drug to prepare it for further pharmaceutical operation. [L. *insuco*, pp. *-atus*, to soak in, fr. *in*, in, + *sucus*, juice, sap (improp. *succ*)]

**in-su-date** (in'soo-dät). Fluid swelling within an arterial wall (ordinarily serous), differing from an exudate in that it does not come to lie extramurally. [L. *in*, in, + *sud*, pp. *-atus*, to sweat]

**in-su-fla-cien-cy** (in-sü-fish'ë-në). Lack of completeness of function or power. *SEE ALSO* incompetence. [L. *in*, neg. + *sufficiens*, fr. *sufficere* to suffice]

**accommodative** *i.*, a lack of appropriate accommodation for near focus.

**acute adrenocortical** *i.*, severe adrenocortical *i.* when an intercurrent illness or trauma causes an increased demand for adrenocortical hormones in a patient with adrenal insufficiency due to disease or use of relatively large amounts of similar hormones as therapy; characterized by nausea, vomiting, hypotension, and frequently hyperthermia, hyponatremia, hyperkalemia, and hypoglycemia; can be fatal if untreated. *syn* addisonian crisis, adrenal crisis, Bernard-Souvenard syndrome.

**adrenocortical** *i.*, loss, to varying degrees, of adrenocortical function. *syn* hypocorticism.

**aortic** *i.*, *SEE* valvular regurgitation.

**cardiac** *i.*, *syn* heart failure (1).

**chronic adrenocortical** *i.*, adrenocortical *i.* usually as the result of idiopathic atrophy or destruction of both adrenal glands by tuberculosis, an autoimmune process, or other diseases; characterized by fatigue, decreased blood pressure, weight loss, increased melanin pigmentation of the skin and mucous membranes, anorexia, and nausea or vomiting; without appropriate replacement therapy, it can progress to acute adrenocortical *i.* *syn* Addison disease, addisonian syndrome, hypoparadrenism, morbus Addison.

**convergence** *i.*, that condition in which an exophoria or exotropia is more marked for near vision than for far vision.

M. "Substance abuse disorder" (PubMed search) – entered November 3, 2006



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<p>(54) Title: COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES</p> <p>(57) Abstract</p> <p>Compounds, compositions, and methods are provided for treatment of disorders related to mitochondrial dysfunction. The methods comprise administering to a mammal a composition containing pyrimidien nucleotide precursors in amounts sufficient to treat symptoms resulting from mitochondrial respiratory chain deficiencies.</p>			

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## COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES

### Field of the Invention

This invention relates generally to compounds and methods for treatment and prevention of diseases, developmental delays, and symptoms related to mitochondrial dysfunction. Pyrimidine nucleotide precursors are administered to a mammal, including a human, for the purpose of compensating for mitochondrial dysfunction and for improving mitochondrial functions.

### Background of the Invention

Mitochondria are cellular organelles present in most eukaryotic cells. One of their primary functions is oxidative phosphorylation, a process through which energy derived from metabolism of fuels like glucose or fatty acids is converted to ATP, which is then used to drive various energy-requiring biosynthetic reactions and other metabolic activities. Mitochondria have their own genomes, separate from nuclear DNA, comprising rings of DNA with about 16,000 base pairs in human cells. Each mitochondrion may have multiple copies of its genome, and individual cells may have hundreds of mitochondria.

Mitochondrial dysfunction contributes to various disease states. Some mitochondrial diseases are due to mutations or deletions in the mitochondrial genome. Mitochondria divide and proliferate with a faster turnover rate than their host cells, and their replication is under control of the nuclear genome. If a threshold proportion of mitochondria in a cell is defective, and if a threshold proportion of such cells within a tissue have defective mitochondria, symptoms of tissue or organ dysfunction can result. Practically any tissue can be affected, and a large variety of symptoms may be present, depending on the extent to which different tissues are involved.

A fertilized ovum might contain both normal and genetically defective mitochondria. The segregation of defective mitochondria into different tissues during division of this ovum is a stochastic process, as will be the ratio of defective to normal mitochondria within a given tissue or cell (although there can be positive or negative selection for defective mitochondrial genomes during mitochondrial turnover within cells). Thus, a variety of different pathologic phenotypes can emerge out of a particular point mutation in mitochondrial DNA. Conversely, similar phenotypes can emerge from mutations or deletions affecting different genes within mitochondrial DNA. Clinical symptoms in congenital mitochondrial diseases often manifest in postmitotic tissues with high energy demands like brain, muscle, optic nerve, and myocardium, but other tissues including endocrine glands, liver, gastrointestinal tract, kidney, and hematopoietic tissue are also involved, again depending in part on the segregation of mitochondria during development, and on the dynamics of mitochondrial turnover over time.

In addition to congenital disorders involving inherited defective mitochondria, acquired mitochondrial dysfunction contributes to diseases, particularly neurodegenerative disorders associated with aging like Parkinson's, Alzheimer's, Huntington's Diseases. The incidence of somatic mutations in mitochondrial DNA rises exponentially with age; diminished respiratory chain activity is found universally in aging people. Mitochondrial dysfunction is also implicated in excitotoxic neuronal injury, such as that associated with seizures or ischemia.

Treatment of diseases involving mitochondrial dysfunction has heretofore involved administration of vitamins and cofactors used by particular elements of the mitochondrial respiratory chain. Coenzyme Q (ubiquinone), nicotinamide, riboflavin, carnitine, biotin, and lipoic acid are used in patients with mitochondrial disease, with occasional benefit, especially in disorders directly stemming from primary deficiencies of one of these cofactors. However, while useful in isolated cases, no such metabolic cofactors or vitamins have been shown to have general utility in clinical practice in treating mitochondrial diseases. Similarly, dichloracetic acid (DCA) has been used to treat mitochondrial cytopathies such as MELAS; DCA inhibits lactate formation and is primarily useful in cases of mitochondrial diseases where excessive lactate accumulation itself is contributing to symptoms. However, DCA does not address symptoms related to mitochondrial insufficiency per se and can be toxic to some patients, depending on the underlying molecular defects.

Mitochondrial diseases comprise disorders caused by a huge variety of molecular lesions or defects, with the phenotypic expression of disease further complicated by stochastic distributions of defective mitochondria in different tissues.

Commonly owned United States Patent 5,583,117 discloses acylated derivatives of cytidine and uridine. Commonly owned application PCT/US 96/10067 discloses the use of acylated pyrimidine nucleosides to reduce the toxicity of chemotherapeutic and antiviral pyrimidine nucleoside analogs.

#### Objects of the Invention

It is an object of the invention to provide compositions and methods for treating disorders or pathophysiological consequences associated with mitochondrial dysfunction or mitochondrial respiratory chain dysfunction in a mammal, including a human.

It is an object of the invention to provide compounds and compositions that improve tissue resistance to mitochondrial dysfunction in vivo.

It is an object of the invention to provide compositions and methods for treatment of mitochondrial diseases.

It is an object of the invention to provide agents which compensate broadly for mitochondrial deficits involving a wide variety of molecular pathologies, since, in many cases, precise diagnosis of molecular lesions in mitochondrial disorders is difficult.

It is an object of the invention to provide a practical treatment for mitochondrial diseases that is beneficial in the case of mitochondrial electron transport chain deficits regardless of the specific molecular defects.

It is an object of the invention to provide not only for the relatively rare congenital diseases related to mitochondrial DNA defects, but also for significant neuromuscular and

neurodevelopmental disorders that appear in childhood and for common age-related degenerative diseases like Alzheimer's or Parkinson's Diseases.

It is an object of the invention to provide compositions and methods for treatment and prevention of neurodegenerative and neuromuscular disorders.

It is an object of the invention to provide compositions and methods for treatment and prevention of excitotoxic injury to neural tissue.

It is an object of the invention to provide compositions and methods for treatment and prevention of epilepsy.

It is an object of the invention to provide compositions and methods for treatment and prevention of migraine.

It is an object of the invention to provide compositions and methods for preventing death or dysfunction of postmitotic cells in a mammal, including a human.

It is an object of the invention to provide compositions and methods for treatment of neurodevelopmental delay disorders

It is a further object of the invention to provide a composition for treatment or prevention of tissue damage due to hypoxia or ischemia .

It is a further object of this invention to provide compositions and methods for treating or preventing ovarian dysfunction, menopause, or secondary consequences of menopause.

It is a further object of the invention to provide compositions and methods for reducing side effects of cancer chemotherapies due to chemotherapy-induced mitochondrial injury.

It is a further object of the invention to provide a method for diagnosing mitochondrial disease and dysfunction.

### Summary of the Invention

The subject invention provides a method for treating pathophysiological consequences of mitochondrial respiratory chain deficiency in a mammal comprising administering to such a mammal in need of such treatment an amount of a pyrimidine nucleotide precursor effective in reducing the pathophysiological consequences. Additionally, the invention provides a method of preventing pathophysiological consequences of mitochondrial respiratory chain deficiency comprising administering to a mammal an amount of a pyrimidine nucleotide precursor effective in preventing the pathophysiological consequences.

In mitochondrial disease the compounds and compositions of the invention are useful for attenuating clinical sequelae stemming from respiratory chain deficiencies. Respiratory chain deficiencies underlying mitochondrial disease are caused by various factors including congenital or inherited mutations anddeletions in mitochondrial DNA, deficits in nuclear-encoded proteins affecting respiratory chain activity, as well as somatic mutations, elevated intracellular calcium, excitotoxicity, nitric oxide, hypoxia and axonal transport defects.

The subject invention provides compounds, compositions, and methods for preventing or reducing death and dysfunction of postmitotic cells bearing mitochondrial respiratory chain deficits.

The subject invention furthermore provides compounds, compositions, and methods for treating neurodevelopmental delays in language, motor, executive function, cognitive, and neuropsychological social skills.

The subject invention also relates to treatment of disorders and conditions that are herein disclosed as conditions to which mitochondrial defects contribute and which therefore are subject to treatment with compounds, and compositions of the invention. These include side effects of cancer chemotherapy like peripheral neuropathies, nephropathies, fatigue, and early menopause, as well as ovulatory abnormalities and normal menopause itself.

The subject invention also relates to a method for diagnosing mitochondrial diseases by treating patients with a pyrimidine nucleotide precursor and assessing clinical benefit in - selected signs and symptoms.

The invention, as well as other objects, features and advantages thereof will be understood more clearly and fully from the following detailed description, when read with reference to the accompanying results of the experiments discussed in the examples below.

#### **Detailed Description of the Invention**

The subject invention is related to compounds, compositions, and methods for treating or preventing a variety of clinical disorders secondary to mitochondrial dysfunction, especially deficits in the activity of components of the mitochondrial respiratory chain. Such disorders include congenital mitochondrial cytopathies, neurodevelopmental delays, age-related neurodegenerative diseases, as well as particular diseases affecting the heart, peripheral and autonomic nerves, skeletal muscle, pancreas and other tissues and organs.

#### **A. Definitions**

"Mitochondrial disease" refers to disorders to which deficits in mitochondrial respiratory chain activity contribute in the development of pathophysiology of such disorders in a mammal. This category includes 1) congenital genetic deficiencies in activity of one or more components of the mitochondrial respiratory chain; 2) acquired deficiencies in the activity of one or more components of the mitochondrial respiratory chain, wherein such deficiencies are caused by, inter alia, a) oxidative damage during aging; b) elevated intracellular calcium; c) exposure of affected cells to nitric oxide; d) hypoxia or ischemia; e) microtubule-associated deficits in axonal transport of mitochondria, or f) expression of mitochondrial uncoupling proteins.

The mitochondrial respiratory chain (also known as the electron transport chain) comprises 5 major complexes:

- Complex I NADH:ubiquinone reductase
- Complex II Succinate:ubiquinone reductase
- Complex III ubiquinol:cytochrome-c reductase
- Complex IV cytochrome-c oxidase
- Complex V ATP synthase

Complexes I and II accomplish the transfer of electrons from metabolic fuels like glycolysis products and fatty acids to ubiquinone (Coenzyme Q), converting it to ubiquinol. Ubiquinol is converted back to ubiquinone by transfer of electrons to cytochrome c in Complex III. Cytochrome c is reoxidized at Complex IV by transfer of electrons to molecular oxygen, - producing water. Complex V utilizes potential energy from the proton gradient produced across the mitochondrial membrane by these electron transfers, converting ADP into ATP, which then provides energy to metabolic reactions in the cell.

Dihydro-orotate dehydrogenase (DHODH), is an enzyme involved in de novo synthesis of uridine nucleotides. DHODH activity is coupled to the respiratory chain via transfer of electrons from dihydro-orotate to ubiquinone; these electrons are then passed onto cytochrome c and oxygen via Complexes III and IV respectively. Only Complexes III and IV are directly involved in pyrimidine biosynthesis. Orotate produced by the action of DHODH is converted to uridine monophosphate by phosphoribosylation and decarboxylation.

"Pyrimidine nucleotide precursors" in the context of the invention are intermediates in either the de novo or salvage pathways of pyrimidine nucleotide synthesis that enter into - pyrimidine synthesis either distal to DHODH (e.g. orotate) or which do not require DHODH activity for conversion to pyrimidine nucleotides (e.g. cytidine, uridine, or acyl derivatives of cytidine or uridine). Also included within the scope of the invention are pyrimidine nucleoside phosphates (e.g. nucleotides, cytidine diphosphocholine, uridine diphosphoglucose); these compounds are degraded to the level of uridine or cytidine prior to entry into cells and anabolism. Acyl derivatives of cytidine and uridine have better oral bioavailability than the parent nucleosides or nucleotides. Orotic acid and esters thereof are converted to uridine nucleotides and are also useful for accomplishing the goals of the invention.

B. Compounds of the Invention

A primary feature of the present invention is the unexpected discovery that administration of pyrimidine nucleotide precursors is effective in treatment of a large variety of symptoms and disease states related to mitochondrial dysfunction.

Tissue pyrimidine nucleotide levels are increased by administration of any of several precursors. Uridine and cytidine are incorporated into cellular nucleotide pools by phosphorylation at the 5' position; cytidine and uridine nucleotides are interconvertible through enzymatic amination and de-amination reactions. Orotic acid is a key intermediate in de novo biosynthesis of pyrimidine nucleotides. Incorporation of orotic acid into nucleotide pools requires cellular phosphoribosyl pyrophosphate (PRPP). Alternatively (or in addition to provision of exogenous nucleotide precursors), availability of uridine to tissues is increased by administration of compounds which inhibit uridine phosphorylase, the first enzyme in the pathway for degradation of uridine. The compounds of the invention useful in treating mitochondrial diseases and related disorders include uridine, cytidine, orotate, orally bioavailable acyl derivatives or esters of these pyrimidine nucleotide precursors, and inhibitors of the enzyme uridine phosphorylase.

In reference to acyl derivatives of cytidine and uridine, the following definitions pertain:

The term "acyl derivative" as used herein means a derivative of a pyrimidine nucleoside in which a substantially nontoxic organic acyl substituent derived from a carboxylic acid is attached to one or more of the free hydroxyl groups of the ribose moiety of the oxy-purine nucleoside with an ester linkage and/or where such a substituent is attached to the amine substituent on the purine ring of cytidine, with an amide linkage. Such acylsubstituents are derived from carboxylic acids which include, but are not limited to, compounds selected from the group consisting of a fatty acid, an amino acid, nicotinic acid, di-carboxylic acids, lactic acid, p-aminobenzoic acid and orotic acid. Advantageous acyl substituents are compounds which are normally present in the body, either as dietary constituents or as intermediary metabolites.

The term "pharmaceutically acceptable salts" as used herein means salts with pharmaceutically acceptable acid or base addition salts of the derivatives, which include, but are not limited to, sulfuric, hydrochloric, or phosphoric acids, or, in the case of orotate, sodium or calcium hydroxides, and cationic amino acids, especially lysine.

The term "amino acids" as used herein includes, but is not limited to, glycine, the L-forms of alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, proline, hydroxyproline, -serine, threonine, cysteine, cystine, methionine, tryptophan, aspartic acid, glutamic acid, arginine, lysine, histidine, ornithine, hydroxylysine, camitine, and other naturally occurring amino acids.

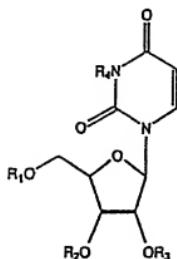
The term "fatty acids" as used herein means aliphatic carboxylic acids having 2-22 carbon atoms. Such fatty acids may be saturated, partially saturated or polyunsaturated.

The term "dicarboxylic acids" as used herein means fatty acids with a second carboxylic acid substituent.

Compounds of the invention have the following structures:

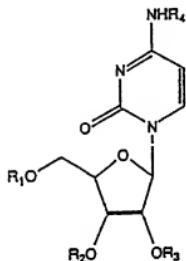
In all cases except where indicated, letters and letters with subscripts symbolizing variable substituents in the chemical structures of the compounds of the invention are applicable only to the structure immediately preceding the description of the symbol.

(1) An acyl derivative of uridine having the formula:



wherein R1, R2, R3 and R4 are the same or different and each is hydrogen or an acyl radical of a metabolite, provided that at least one of said R substituents is not hydrogen, or a pharmaceutically acceptable salt thereof.

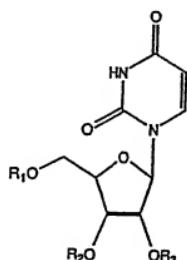
(2) An acyl derivative of cytidine having the formula:



wherein R1, R2, R3 and R4 are the same or different and each is hydrogen or an acyl radical of a metabolite, provided that at least one of said R substituents is not hydrogen, or a pharmaceutically acceptable salt thereof.

The compounds of the invention useful in treating mitochondrial diseases include:

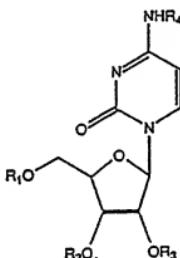
(3) An acyl derivative of uridine having the formula:



wherein R1, R2, and R3 are the same, or different, and each is hydrogen or an acyl radical of

- a. an unbranched fatty acid with 2 to 22 carbon atoms,
- b. an amino acid selected from the group consisting of glycine, the L forms of alanine, valine, leucine, isoleucine, tyrosine, proline, hydroxyproline, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, arginine, lysine, histidine, carnitine and ornithine,
- c. a dicarboxylic acid having 3-22 carbon atoms,
- d. a carboxylic acid selected from one or more of the group consisting of glycolic acid, pyruvic acid, lactic acid, enolpyruvic acid, lipoic acid, pantothenic acid, acetoacetic acid, p-aminobenzoic acid, betahydroxybutyric acid, orotic acid, and creatine.

(4) An acyl derivatives of cytidine having the formula:



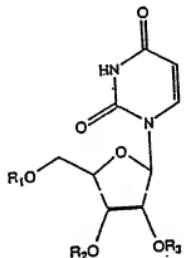
wherein R1, R2, R3, and R4 are the same, or different, and each is hydrogen or an acyl radical

of

- a. an unbranched fatty acid with 2 to 22 carbon atoms,
- b. an amino acid selected from the group consisting of glycine, the L forms of phenylalanine, alanine, valine, leucine, isoleucine, tyrosine, proline, hydroxyproline, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, arginine, lysine, histidine carnitine and ornithine,
- c. a dicarboxylic acid having 3-22 carbon atoms,

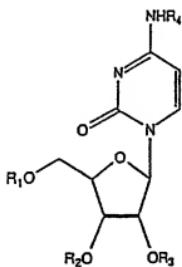
d. a carboxylic acid selected from one or more of the group consisting of glycolic acid, pyruvic acid, lactic acid, enolpyruvic acid, lipoic acid, pantothenic acid, acetoacetic acid, p-aminobenzoic acid, betahydroxybutyric acid, orotic acid, and creatine.

(5) An acyl derivative of uridine having the formula:



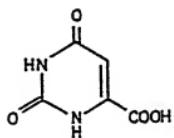
wherein at least one of R1, R2, or R3 is a hydrocarboloxycarbonyl moiety containing 2-26 carbon atoms and the remaining R substituents are independently a hydrocarboloxycarbonyl or hydrocarbonylcarbonyl moiety or H or phosphate.

(6) An acyl derivative of cytidine having the formula:



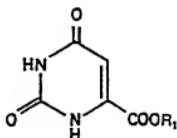
wherein at least one of R1, R2, R3 or R4 is a hydrocarbyloxycarbonyl moiety containing 2-26 carbon atoms and the remaining R substituents are independently a hydrocarbyloxycarbonyl or hydrocarbylcarbonyl moiety or H or phosphate.

(7) Orotic acid or salts thereof:



Pharmaceutically-acceptable salts of orotic acid include those in which the cationic component of the salt is sodium, potassium, a basic amino acid such as arginine or lysine, methylglucamine, choline, or any other substantially nontoxic water soluble cation with a molecular weight less than about 1000 daltons.

8) Alcohol-substituted orotate derivatives:



wherein R1 is a radical of an alcohol containing 1 to 20 carbon atoms joined to orotate via an ester linkage.

Also encompassed by the invention are the pharmaceutically acceptable salts of the above-noted compounds.

Advantageous compounds of the invention are short-chain (2 to 6 carbon atoms) fatty acid esters of uridine or cytidine. Particularly advantageous compounds are triacetyluridine or triacetylcytidine. Such compounds have better oral bioavailability than the parent nucleosides, and are rapidly deacetylated following absorption after oral administration.

Pyruvic acid is useful for treatment of cells with defective mitochondrial function. Cells with reduced capability for mitochondrial oxidative phosphorylation must rely on glycolysis for generation of ATP. Glycolysis is regulated by the redox state of cells. Specifically, NAD<sup>+</sup> is required for optimal glucose flux, producing NADH in the process. In order to maximize energy production from glycolysis, NADH must be reoxidized to NAD<sup>+</sup>. Exogenous pyruvate can reoxidize NADH, in part via a plasma membrane enzyme, NADH Oxidase.

Uridine tripyruvate (2',3',5'-tri-O-pyruvyluridine) provides the benefits of both pyrimidines and pyruvate, delivering both with a single chemical entity, and avoiding the load of sodium, calcium, or other cations in the corresponding salts of pyruvic acid.

#### Inhibitors of uridine phosphorylase

An alternative or complementary strategy for treating mitochondrial diseases involves inhibition of uridine catabolism with an inhibitor of the enzyme uridine phosphorylase.

Examples of inhibitors of uridine phosphorylase that are useful for treatment of mitochondrial disease include but are not limited to 5-benzyl barbiturate or 5-benzylidene barbiturate derivatives including 5-benzyl barbiturate, 5-benzyloxybenzylbarbiturate, 5-benzyloxybenzyl-1-[(1-hydroxy-2-ethoxy)methyl]barbiturate, 5-benzyloxybenzylacetyl-1-[(1-hydroxy-2-ethoxy)methyl] barbiturate, and 5-methoxybenzylacetyl-acyclobarbiturate, 2,2'-

anhydro-5-ethyluridine, 5-ethyl-2-deoxyuridine and acyclouridine compounds, particularly 5-benzyl substituted acyclouridine congeners including but not limited to benzylacyclouridine, benzylxyloxy-benzyl-acyclo-uridine, aminomethyl-benzyl-acyclouridine, aminomethyl-benzylxyloxy-benzylacyclouridine, hydroxymethyl-benzylacyclouridine, and hydroxymethyl-benzylxyloxy-benzyl-acyclouridine. See also WO 89/09603 and WO 91/16315, hereby incorporated by reference.

#### C. Compositions of the Invention

In one embodiment of the invention, novel pharmaceutical compositions comprise as an active agent one or more pyrimidine nucleotide precursors selected from the group consisting of uridine, cytidine, orotic acid or its salts or esters, and acyl derivatives of these pyrimidine nucleotide precursors, together with a pharmaceutically acceptable carrier.

The compositions, depending on the intended use and route of administration, are manufactured in the form of a liquid, a suspension, sprinkles, microcapsules, a tablet, a capsule, a dragee, an injectable solution, or a suppository (see discussion of formulation below).

In another embodiment of the invention, the composition comprises at least one pyrimidine nucleotide precursor and an agent which inhibits the degradation of uridine, such as an inhibitor of the enzyme uridine phosphorylase. Examples of inhibitors of uridine phosphorylase include but are not limited to 5-benzyl barbiturate or 5-benzylidene barbiturate derivatives including 5-benzyl barbiturate, 5-benzylxyloxybenzyl barbiturate, 5-benzylxyloxybenzyl-1-[(1-hydroxy-2-ethoxy)methyl] barbiturate, 5-benzylxyloxybenzylacetyl-1-[(1-hydroxy-2-ethoxy)methyl] barbiturate, and 5-methoxybenzylacetyl-acyclobarbiturate, 2,2'-anhydro-5-ethyluridine, and acyclouridine compounds, particularly 5-benzyl substituted acyclouridine congeners including but not limited to benzylacyclouridine, benzylxyloxy-benzyl-acyclo-uridine, aminomethyl-benzyl-acyclouridine, aminomethyl-benzylxyloxy-benzyl-acyclouridine, hydroxymethyl-benzylacyclouridine, and hydroxymethyl-benzylxyloxy-benzyl-acyclouridine. Furthermore, it is within the scope of the invention to utilize an inhibitor of uridine phosphorylase alone, without coadministration of a pyrimidine nucleotide precursor, for the

purpose of treating mitochondrial diseases or pathophysiologies associated with mitochondrial respiratory chain dysfunction.

Further embodiments of the invention comprise a pyrimidine nucleotide precursor combined with one or more other agents with protective or supportive activity relative to mitochondrial structure and function. Such agents, presented with recommended daily doses in mitochondrial diseases include, but are not limited to, pyruvate (1 to 10 grams/day), Coenzyme Q (1 to 4mg/kg/day), alanine (1-10 grams/day), lipoic acid (1 to 10mg/kg/day), carnitine (10 to 100 mg/kg/day), riboflavin (20 to 100 mg/day), biotin (1 to 10 mg/day), nicotinamide (20 to 100 mg/day), niacin (20 to 100 mg/day), Vitamin C (100 to 1000mg/day), Vitamin E (200-400 mg/day), and dichloroacetic acid or its salts. In the case of pyruvate, this active agent can be administered as pyruvic acid, pharmaceutically acceptable salts thereof, or pyruvic acid esters having an alcohol moiety containing 2 to 10 carbon atoms.

#### D. Therapeutic Uses of the Compounds and Compositions of the Invention

Diseases related to mitochondrial respiratory chain dysfunction can be divided into several categories based on the origin of mitochondrial defects.

Congenital mitochondrial diseases are those related to hereditary mutations, deletions, or other defects in mitochondrial DNA or in nuclear genes regulating mitochondrial DNA integrity, or in nuclear genes encoding proteins that are critical for mitochondrial respiratory chain function.

Acquired mitochondrial defects comprise primarily 1) damage to mitochondrial DNA due to oxidative processes or aging; 2) mitochondrial dysfunction due to excessive intracellular and intramitochondrial calcium accumulation; 3) inhibition of respiratory chain complexes with endogenous or exogenous respiratory chain inhibitors; 4) acute or chronic oxygen deficiency; and 5) impaired nuclear-mitochondrial interactions, e.g. impaired shuttling of mitochondria in long axons due to microtubule defects, and 6) expression of mitochondrial uncoupling proteins in response to lipids, oxidative damage or inflammation.

The most fundamental mechanisms involved in acquired mitochondrial defects, and which underlie pathogenesis of a variety of forms of organ and tissue dysfunction, include:

**Calcium accumulation:** A fundamental mechanism of cell injury, especially in excitable tissues, involves excessive calcium entry into cells, as a result of either leakage through the plasma membrane or defects in intracellular calcium handling mechanisms. Mitochondria are major sites of calcium sequestration, and preferentially utilize energy from the respiratory chain for taking up calcium rather than for ATP synthesis, which results in a downward spiral of mitochondrial failure, since calcium uptake into mitochondria results in diminished capabilities for energy transduction.

**Excitotoxicity:** Excessive stimulation of neurons with excitatory amino acids is a common mechanism of cell death or injury in the central nervous system. Activation of glutamate receptors, especially of the subtype designated NMDA receptors, results in mitochondrial dysfunction, in part through elevation of intracellular calcium during excitotoxic stimulation. Conversely, deficits in mitochondrial respiration and oxidative phosphorylation sensitizes cells to excitotoxic stimuli, resulting in cell death or injury during exposure to levels of excitotoxic neurotransmitters or toxins that would be innocuous to normal cells.

**Nitric oxide exposure:** Nitric oxide (~1 micromolar) inhibits cytochrome oxidase (Complex IV) and thereby inhibits mitochondrial respiration (Brown GC, Mol. Cell. Biochem. 174:189-192, 1997); moreover, prolonged exposure to NO irreversibly reduces Complex I activity. Physiological or pathophysiological concentrations of NO thereby inhibit pyrimidine biosynthesis. Nitric oxide is implicated in a variety of neurodegenerative disorders including inflammatory and autoimmune diseases of the central nervous system, and is involved in mediation of excitotoxic and post-hypoxic damage to neurons.

**Hypoxia:** Oxygen is the terminal electron acceptor in the respiratory chain. Oxygen deficiency impairs electron transport chain activity, resulting in diminished pyrimidine synthesis as well as diminished ATP synthesis via oxidative phosphorylation. Human cells proliferate and retain viability under virtually anaerobic conditions if provided with uridine and pyruvate (or a similarly effective agent for oxidizing NADH to optimize glycolytic ATP production).

**Nuclear-mitochondrial interactions:** Transcription of mitochondrial DNA encoding respiratory chain components requires nuclear factors. In neuronal axons, mitochondria must shuttle back and forth to the nucleus in order to maintain respiratory chain activity. If axonal transport is impaired by hypoxia or by drugs like taxol which affect microtubule stability, mitochondria distant from the nucleus undergo loss of cytochrome oxidase activity.

**Mitochondrial Uncoupling Proteins:** Mitochondria are the primary source of free radicals and reactive oxygen species, due to spillover from the mitochondrial respiratory chain, especially when defects in one or more respiratory chain components impairs orderly transfer of electrons from metabolic intermediates to molecular oxygen. To reduce oxidative damage, cells can compensate by expressing mitochondrial uncoupling proteins (UCP), of which several have been identified. UCP-2 is transcribed in response to oxidative damage, inflammatory cytokines, or excess lipid loads, e.g. fatty liver and steatohepatitis. UCP reduce spillover of reactive oxygen species from mitochondria by discharging proton gradients across the mitochondrial inner membrane, in effect wasting energy produced by metabolism and rendering cells vulnerable to energy stress as a trade-off for reduced oxidative injury.

In the nervous system especially, mitochondrial respiratory chain deficits have two generalizable consequences: 1) Delayed or aberrant development of neuronal circuits within the nervous system; and 2) accelerated degeneration of neurons and neural circuits, either acutely or over a period of years, depending on the severity of the mitochondrial deficits and other-precipitating factors. Analogous patterns of impaired development and accelerated degeneration pertain to non-neural tissues and systems as well.

#### *Mitochondrial dysfunction and pyrimidine biosynthesis*

Cells with severely damaged mitochondria (including total deletion of mitochondrial DNA, with a consequent shutdown of respiratory chain activity) can survive in culture if provided with two agents which compensate for critical mitochondrial functions: uridine and pyruvate. Uridine is required *in vitro* because a limiting enzyme for *de novo* synthesis of uridine nucleotides, dihydro-orotate dehydrogenase (DHODH), is coupled to the mitochondrial

respiratory chain, via ubiquinone as a proximal electron acceptor, cytochrome c as an intermediate, and oxygen as a terminal electron acceptor (Loffler et al., Mol. Cell. Biochem. 174:125-129, 1997). DHODH is required for synthesis of orotate, which is then phosphoribosylated and decarboxylated to produce uridine monophosphate (UMP). All other pyrimidines in cells are derived from UMP. Cells from patients with mitochondrial disease due to defects in mitochondrial DNA require exogenous uridine in order to survive outside of the milieu of the body, wherein pyrimidines, derived from other cells or the diet, and transported via the circulation, are *prima facie* sufficient to support their viability (Bourgeron, et al. Neuromusc. Disord. 3:605-608, 1993). Significantly, intentional inhibition of DHODH with drugs like Brequinar or Leflunomide results in dose-limiting cytotoxic damage to the hematopoietic system and gastrointestinal mucosa, in contrast to the predominant involvement of postmitotic tissues like the nervous system and muscle in clinical mitochondrial disease.

*Pathophysiological consequences of respiratory chain dysfunction*

Mitochondria are critical for the survival and proper function of almost all types of eukaryotic cells. Mitochondria in virtually any cell type can have congenital or acquired defects that affect their function. Thus, the clinically significant signs and symptoms of mitochondrial defects affecting respiratory chain function are heterogeneous and variable depending on the distribution of defective mitochondria among cells and the severity of their deficits, and upon physiological demands upon the affected cells. Nondividing tissues with high energy requirements, e.g. nervous tissue, skeletal muscle and cardiac muscle are particularly susceptible to mitochondrial respiratory chain dysfunction, but any organ system can be affected.

The diseases and symptoms listed below comprise known pathophysiological consequences of mitochondrial respiratory chain dysfunction and as such are disorders in which the compounds and compositions of the invention have therapeutic utility.

Disease symptoms secondary to mitochondrial dysfunction are generally attributed to 1) spillover of free radicals from the respiratory chain; 2) deficits in ATP synthesis leading to cellular energy failure, or 3) apoptosis triggered by release of mitochondrial signals like

cytochrome c which initiate or mediate apoptosis cascades. An unexpected feature of the instant invention is the observation that pyrimidine nucleotide precursors of the invention have therapeutic activity against a large variety of symptoms in patients with mitochondrial disease, as shown in the Examples. This constitutes an important paradigm shift in the understanding of pathogenesis of diseases involving mitochondrial dysfunction, and in understanding how to treat such disorders.

Treatment of congenital mitochondrial cytopathies

*Mitochondrial DNA defects*

A number of clinical syndromes have been linked to mutations or deletions in mitochondrial DNA. Mitochondrial DNA is inherited maternally, with virtually all of the mitochondria in the body derived from those provided by the oocyte. If there is a mixture of defective and normal mitochondria in an oocyte, the distribution and segregation of mitochondria is a stochastic process. Thus, mitochondrial diseases are often multisystem disorders, and a particular point mutation in mitochondrial DNA, for example, can result in dissimilar sets of signs and symptoms in different patients. Conversely, mutations in two different genes in mitochondrial DNA can result in similar symptom complexes.

Nonetheless, some consistent symptom patterns have emerged in conjunction with identified mitochondrial DNA defects, and these comprise the classic "mitochondrial diseases", some of which are listed immediately below. Nonetheless, an important aspect of the subject invention is the recognition that the concept of mitochondrial disease and its treatment with compounds and compositions of the invention extends to many other disease conditions which are also disclosed herein.

Some of the classical phenotypes of major mitochondrial diseases associated with mutations or deletions of mitochondrial DNA include:

MELAS: (Mitochondrial Encephalomyopathy Lactic Acidemia, and Stroke-like episodes.

MERRF: Myoclonic Epilepsy with "Ragged Red" (muscle) Fibers

MNGIE: Mitochondrial neurogastrointestinal encephalomyopathy

NARP: Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa

LHON: Leber's Hereditary Optic Neuropathy

Leigh's Syndrome (Subacute Necrotizing Encephalomyopathy)

PEO: Progressive External Ophthalmoplegia

Kearns-Sayres Syndrome (PEO, pigmentary retinopathy, ataxia, and heart-block)

Other common symptoms of mitochondrial diseases which may be present alone or in conjunction with these syndromes include cardiomyopathy, muscle weakness and atrophy, developmental delays(involving motor, language, cognitive or executive function),ataxia, epilepsy, renal tubular acidosis, peripheral neuropathy,optic neuropathy, autonomic neuropathy, neurogenic bowel dysfunction, sensorineural deafness, neurogenic bladder dysfunction, dilating cardiomyopathy, migraine, hepatic failure, lactic acidemia, and diabetes mellitus.

In addition, gene products and tRNA encoded by mitochondrial DNA, many proteins involved in, or affecting, mitochondrial respiration and oxidative phosphorylation are encoded by nuclear DNA. In fact, approximately 3000 proteins, or 20% of all proteins encoded by the nuclear genome, are physically incorporated into, or associated with, mitochondria and mitochondrial functions or biogenesis, although only about 100 are directly involved as structural components of the respiratory chain. Therefore, mitochondrial diseases involve not only gene products of mitochondrial DNA, but also nuclear encoded proteins affecting respiratory chain function and mitochondrial structure.

Metabolic stressors like infections can unmask mitochondrial defects that do not necessarily yield symptoms under normal conditions. Neuromuscular or neurological setbacks during infection are a hallmark of mitochondrial disease. Conversely, mitochondrial respiratory chain dysfunction can render cells vulnerable to stressors that would otherwise be innocuous.

Diagnosis of congenital mitochondrial disease is challenging, due to the heterogeneity of symptoms, even between patients affected with the same molecular defect. Deficits in cell and tissue function due to mitochondrial dysfunction can mimic tissue dysfunction caused by problems that do not directly involve mitochondrial defects. Several clinically useful and practical schemes for diagnosis of mitochondrial diseases are known in the art; they typically involve several major criteria (e.g. classical clinical phenotypes like MELAS, NARP or Leigh's Syndrome, extreme (>80%) depressions of respiratory chain complex activity in fresh tissue samples) with a good degree of certainty in establishing the role of respiratory chain dysfunction in disease pathogenesis, and a larger number of minor criteria (e.g. moderate biochemical abnormalities characteristic of respiratory chain defects, symptoms characteristic of mitochondrial diseases without full presentation of one of the classical phenotypes listed above) which individually are less compelling than single major criteria, but which cumulatively provide strong evidence for the contribution of respiratory chain deficits to a particular patient's clinical presentation, as described in Walker et al. (Eur Neurol., 36:260-7, 1996), hereby incorporated by reference.

As is demonstrated in the Examples, compounds and compositions of the invention are useful for treatment of a very broad spectrum of signs and symptoms in mitochondrial diseases with different underlying molecular pathologies. Improvements observed in these and additional patients include but are not limited to reduction of frequency and severity of seizures, migraines, and stroke-like episodes, improvement of weight gain in children with "failure to thrive", amelioration of renal tubular acidosis with concurrent reduction in the need for supplementary bicarbonate, improvement of muscular strength, improvement of speech acquisition, improvement of ataxia, reduction of the frequency and severity of sinus and ear infections, improvement of memory, and amelioration of symptoms of autonomic and peripheral neuropathy. The improvements observed in a broad variety of symptoms which were basically nonresponsive to other forms of metabolic support, e.g. vitamins and cofactors known

to be necessary for proper mitochondrial function (which argues against attribution of benefits to a placebo effect, as does recurrence of symptoms when pyrimidine support is withdrawn) demonstrate a major unexpected insight of the invention, that functional or conditional pyrimidine deficiency underlies a wide variety of dominant symptoms in patients with mitochondrial diseases and that pyrimidine supplementation is sufficient to improve or ameliorate a broad variety of symptoms in such patients. Hitherto, symptoms of mitochondrial disease have been attributed to ATP deficiency, reactive oxygen species generated by the defective respiratory chain, or to cell death triggered by mitochondrial components of the apoptosis cascade. The dose limiting toxicity of inhibitors of *de novo* pyrimidine synthesis are typically due to inhibition of proliferation of rapidly dividing cell types like bone marrow and gut mucosal stem cells. Unexpectedly, therapeutic benefits of compounds and methods of the invention in patients and experimental animals have been demonstrated in tissues comprising nondividing postmitotic cells, e.g. central and peripheral neurons and skeletal and cardiac muscle.

An important feature of the subject invention is the unexpected result that treatment of patients with mitochondrial disease caused by a variety of underlying molecular defects results in clinical improvement in a diverse assortment of symptoms *in vivo* in patients (Examples 1-4). It is significant and further unexpected that clinical benefit has been observed even in patients with normal activity of the two respiratory chain complexes (III and IV) that are directly involved in the electron transfers specifically required for pyrimidine biosynthesis.

Furthermore, it is an unexpected and an important aspect of the invention that higher doses of pyrimidine nucleotide precursors of the invention are typically required for optimal treatment effects in patients with mitochondrial cytopathies than are required for adequate treatment of patients with a virtually complete block in *de novo* pyrimidine synthesis, e.g. homozygotes for Type I orotic aciduria. Optimum doses of a compound of the invention, e.g. triacetyluridine (which is efficiently absorbed after oral administration), for treatment of congenital mitochondrial disease in children are in the range of 1 to 6 grams per m<sup>2</sup> of body surface area (50 to 300 mg/kg, advantageously 100 to 300 mg/kg), whereas total daily *de novo* synthesis of pyrimidines is approximately one gram per day in adults (about 0.5 gram/m<sup>2</sup>).

The broad applicability of the methods of the invention are unexpected and set the compounds and compositions of the invention apart from other therapies of mitochondrial disease that have been attempted e.g. Coenzyme Q, B vitamins, carnitine, and lipoic acid, which generally address very specific reactions and cofactors involved in mitochondrial function and which are therefore useful only in isolated cases. However, such metabolic interventions with antioxidants and cofactors of respiratory chain complexes are compatible with concurrent treatment with compounds and compositions of the invention, and in fact are used to their best advantage in combination with compounds and compositions of the invention.

Treatment of neuromuscular degenerative disorders

*Friedreich's Ataxia*

A gene defect underlying Friedreich's Ataxia (FA), the most common hereditary ataxia, was recently identified and is designated "frataxin". In FA, after a period of normal development, deficits in coordination develop which progress to paralysis and death, typically between the ages of 30 and 40. The tissues affected most severely are the spinal cord, peripheral nerves, myocardium, and pancreas. Patients typically lose motor control and are confined to wheel chairs, and are commonly afflicted with heart failure and diabetes.

The genetic basis for FA involves GAA trinucleotide repeats in an intron region of the gene encoding frataxin. The presence of these repeats results in reduced transcription and expression of the gene. Frataxin is involved in regulation of mitochondrial iron content. When cellular frataxin content is subnormal, excess iron accumulates in mitochondria, promoting oxidative damage and consequent mitochondrial degeneration and dysfunction.

When intermediate numbers of GAA repeats are present in the frataxin gene intron, the severe clinical phenotype of ataxia may not develop. However, these intermediate-length trinucleotide extensions are found in 25 to 30% of patients with non-insulin dependent diabetes mellitus, compared to about 5% of the nondiabetic population.

Compounds and compositions of the invention are useful for treating patients with disorders related to deficiencies or defects in frataxin, including Friedreich's Ataxia, myocardial dysfunction, diabetes mellitus and complications of diabetes like peripheral neuropathy. Conversely, diagnostic tests for presumed frataxin deficiencies involving PCR tests for GAA intron repeats are useful for identifying patients who will benefit from treatment with compounds and compositions of the invention.

*Muscular Dystrophy*

Muscular dystrophy refers to a family of diseases involving deterioration of neuromuscular structure and function, often resulting in atrophy of skeletal muscle and myocardial dysfunction. In the case of Duchenne muscular dystrophy, mutations or deficits in a specific protein, dystrophin, are implicated in its etiology. Mice with their dystrophin genes inactivated display some characteristics of muscular dystrophy, and have an approximately 50% deficit in mitochondrial respiratory chain activity. A final common pathway for neuromuscular degeneration in most cases is calcium-mediated impairment of mitochondrial function. Compounds and compositions of the invention are useful for reducing the rate of decline in muscular functional capacities and for improving muscular functional status in patients with muscular dystrophy.

*Multiple sclerosis*

Multiple sclerosis (MS) is a neuromuscular disease characterized by focal inflammatory and autoimmune degeneration of cerebral white matter. Periodic exacerbations or attacks are significantly correlated with upper respiratory tract and other infections, both bacterial and viral, indicating that mitochondrial dysfunction plays a role in MS. Depression of neuronal mitochondrial respiratory chain activity caused by Nitric Oxide (produced by astrocytes and other cells involved in inflammation) is implicated as a molecular mechanism contributing to MS.

Compounds and compositions of the invention are useful for treatment of patients with multiple sclerosis, both prophylactically and during episodes of disease exacerbation.

Treatment of disorders of neuronal instability*Treatment of seizure disorders*

Epilepsy is often present in patients with mitochondrial cytopathies, involving a range of seizure severity and frequency, e.g. absence, tonic, atonic, myoclonic, and status epilepticus, occurring in isolated episodes or many times daily.

In patients with seizures secondary to mitochondrial dysfunction, compounds and methods of the invention are useful for reducing frequency and severity of seizure activity.

*Treatment and prevention of migraine*

Metabolic studies on patients with recurrent migraine headaches indicate that deficits in mitochondrial activity are commonly associated with this disorder, manifesting as impaired - oxidative phosphorylation and excess lactate production. Such deficits are not necessarily due to genetic defects in mitochondrial DNA. Migraineurs are hypersensitive to nitric oxide, an endogenous inhibitor of Cytochrome c Oxidase. In addition, patients with mitochondrial cytopathies, e.g. MELAS, often have recurrent migraines.

In patients with recurrent migraine headaches, compounds, compositions, and methods of the invention are useful for prevention and treatment, especially in the case of headaches refractory to ergot compounds or serotonin receptor antagonists.

As demonstrated in Example 1, compounds and compositions of the invention are useful for treatment of migraines associate with mitochondrial dysfunction.

*Treatment of developmental delay*

Delays in neurological or neuropsychological development are often found in children with mitochondrial diseases. Development and remodeling of neural connections requires intensive biosynthetic activity, particularly involving synthesis of neuronal membranes and

myelin, both of which require pyrimidine nucleotides as cofactors. Uridine nucleotides are involved inactivation and transfer of sugars to glycolipids and glycoproteins. Cytidine nucleotides are derived from uridine nucleotides, and are crucial for synthesis of major membrane phospholipid constituents like phosphatidylcholine, which receives its choline moiety from cytidine diphosphocholine. In the case of mitochondrial dysfunction (due to either mitochondrial DNA defects or any of the acquired or conditional deficits like excitotoxic or nitric oxide-mediated mitochondrial dysfunction described above) or other conditions resulting in impaired pyrimidine synthesis, cell proliferation and axonal extension is impaired at crucial stages in development of neuronal interconnections and circuits, resulting in delayed or arrested development of neuropsychological functions like language, motor, social, executive function, and cognitive skills. In autism for example, magnetic resonance spectroscopy measurements of cerebral phosphate compounds indicates that there is global undersynthesis of membranes and membrane precursors indicated by reduced levels of uridine diphospho-sugars, and cytidine nucleotide derivatives involved in membrane synthesis (Minshew et al., *Biological Psychiatry* 33:762-773, 1993).

Disorders characterized by developmental delay include Rett's Syndrome, pervasive developmental delay (or PDD-NOS: "pervasive developmental delay - not otherwise specified" to distinguish it from specific subcategories like autism), autism, Asperger's Syndrome, and Attention Deficit/Hyperactivity Disorder (ADHD), which is becoming recognized as a delay or lag in development of neural circuitry underlying executive functions.

Compounds and compositions of the invention are useful for treating patients with neurodevelopmental delays involving motor, language, executive function, and cognitive skills. Current treatments for such conditions, e.g. ADHD, involve amphetamine-like stimulants that enhance neurotransmission in some affected underdeveloped circuits, but such agents, which may improve control of disruptive behaviors, do not improve cognitive function, as they do not address underlying deficits in the structure and interconnectedness of the implicated neural circuits.

Compounds and compositions of the invention are also useful in the case of other delays or arrests of neurological and neuropsychological development in the nervous system and somatic development in non-neural tissues like muscle and endocrine glands.

*Treatment of neurodegenerative disorders*

The two most significant severe neurodegenerative diseases associated with aging, Alzheimer's Disease (AD) and Parkinson's Disease (PD), both involve mitochondrial dysfunction in their pathogenesis. Complex I deficiencies in particular are frequently found not only in the nigrostriatal neurons that degenerate in Parkinson's disease, but also in peripheral tissues and cells like muscle and platelets of Parkinson's Disease patients.

In Alzheimer's Disease, mitochondrial respiratory chain activity is often depressed, especially Complex IV (Cytochrome c Oxidase). Moreover, mitochondrial respiratory function altogether is depressed as a consequence of aging, further amplifying the deleterious sequelae of additional molecular lesions affecting respiratory chain function.

Other factors in addition to primary mitochondrial dysfunction underlie neurodegeneration in AD, PD, and related disorders. Excitotoxic stimulation and nitric oxide are implicated in both diseases, factors which both exacerbate mitochondrial respiratory chain deficits and whose deleterious actions are exaggerated on a background of respiratory chain dysfunction.

Huntington's Disease also involves mitochondrial dysfunction in affected brain regions, with cooperative interactions of excitotoxic stimulation and mitochondrial dysfunction contributing to neuronal degeneration. In example 8, a compound of the invention, triacetyluridine, prevents neuronal cell death in a murine model of Huntington's disease.

Compounds and compositions of the invention are useful for treating and attenuating progression of age-related neurodegenerative disease including AD and PD.

*Amyotrophic lateral sclerosis*

One of the major genetic defects in patients with Amyotrophic Lateral Sclerosis (ALS; Lou Gehrig's Disease; progressive degeneration of motor neurons, skeletal muscle atrophy, inevitably leading to paralysis and death) is mutation or deficiency in Copper-Zinc Superoxide Dismutase (SOD1), an antioxidant enzyme. Mitochondria both produce and are primary targets for reactive oxygen species. Inefficient transfer of electrons to oxygen in mitochondria is the most significant physiological source of free radicals in mammalian systems. Deficiencies in antioxidants or antioxidant enzymes can result in or exacerbate mitochondrial degeneration. Mice transgenic for mutated SOD1 develop symptoms and pathology similar to those in human ALS. The development of the disease in these animals has been shown to involve oxidative destruction of mitochondria followed by functional decline of motor neurons and onset of clinical symptoms (Kong and Xu, J. Neurosci. 18:3241-3250, 1998). Skeletal muscle from ALS patients has low mitochondrial Complex I activity (Wiedemann et al., J. Neurol. Sci 156:65-72, 1998).

Compounds, compositions, and methods of the invention are useful for treatment of ALS, for reversing or slowing the progression of clinical symptoms.

*Protection against ischemia and hypoxia*

Oxygen deficiency results in both direct inhibition of mitochondrial respiratory chain activity by depriving cells of a terminal electron acceptor for Cytochrome c reoxidation at Complex IV, and indirectly, especially in the nervous system, via secondary post-anoxic excitotoxicity and nitric oxide formation.

In conditions like cerebral anoxia, angina or sickle cell anemia crises, tissues are relatively hypoxic. In such cases, compounds of the invention provide protection of affected tissues from deleterious effects of hypoxia, attenuate secondary delayed cell death, and accelerate recovery from hypoxic tissue stress and injury.

Compounds and compositions of the invention are useful for preventing delayed cell death (apoptosis in regions like the hippocampus or cortex occurring about 2 to 5 days after an episode of cerebral ischemia) after ischemic or hypoxic insult to the brain.

*Renal tubular acidosis*

Acidosis due to renal dysfunction is often observed inpatients with mitochondrial disease, whether the underlying respiratory chain dysfunction is congenital or induced by - ischemia or cytotoxic agents like cisplatin. Renal tubular acidosis often requires administration of exogenous sodium bicarbonate to maintain blood and tissue pH.

In Example 3, administration of a compound of the invention caused an immediate reversal of renal tubular acidosis in a patient with a severe Complex I deficiency. Compounds and compositions of the invention are useful for treating renal tubular acidosis and other forms of renal dysfunction caused by mitochondrial respiratory chain deficits.

*Age-related neurodegeneration and cognitive decline*

During normal aging, there is a progressive decline in mitochondrial respiratory chain function. Beginning about age 40, there is an exponential rise in accumulation of mitochondrial DNA defects in humans, and a concurrent decline in nuclear-regulated elements of mitochondrial respiratory activity.

de Grey (Bioessays, 19:161-167, 1998) discussed mechanisms underlying the observation that many mitochondrial DNA lesions have a selection advantage during mitochondrial turnover, especially in postmitotic cells. The proposed mechanism is that mitochondria with a defective respiratory chain produce less oxidative damage to themselves than do mitochondria with intact functional respiratory chains (mitochondrial respiration is the primary source of free radicals in the body). Therefore, normally-functioning mitochondria accumulate oxidative damage to membrane lipids more rapidly than do defective mitochondria, and are therefore "tagged" for degradation by lysosomes. Since mitochondria within cells have a half life of about 10 days, a selection advantage can result in rapid replacement of functional -

mitochondria with those with diminished respiratory activity, especially in slowly dividing cells. The net result is that once a mutation in a gene for a mitochondrial protein that reduces oxidative damage to mitochondria occurs, such defective mitochondria will rapidly populate the cell, diminishing or eliminating its respiratory capabilities. The accumulation of such cells results in aging or degenerative disease at the organismal level. This is consistent with the progressive mosaic appearance of cells with defective electron transport activity in muscle, with cells almost devoid of Cytochrome c Oxidase (COX) activity interspersed randomly amidst cells with normal activity, and a higher incidence of COX-negative cells in biopsies from older subjects. The organism, during aging, or in a variety of mitochondrial diseases, is thus faced with a situation in which irreplaceable postmitotic cells (e.g. neurons, skeletal and cardiac muscle) must be preserved and their function maintained to a significant degree, in the face of an inexorable progressive decline in mitochondrial respiratory chain function. Neurons with dysfunctional mitochondria become progressively more sensitive to insults like excitotoxic injury. Mitochondrial failure contributes to most degenerative diseases (especially neurodegeneration) that accompany aging.

Congenital mitochondrial diseases often involve early-onset neurodegeneration similar in fundamental mechanism to disorders that occur during aging of people born with normal mitochondria. The demonstration disclosed in the Examples that compounds and compositions of the invention are useful in treatment of congenital or early-onset mitochondrial disease provides direct support for the utility of compounds and compositions of the invention for treatment of age-related tissue degeneration.

Compounds and compositions of the invention are useful for treating or attenuating cognitive decline and other degenerative consequences of aging.

#### *Mitochondria and cancer chemotherapy*

Mitochondrial DNA is typically more vulnerable to damage than is nuclear DNA for several reasons:

1. Mitochondrial DNA has a less sophisticated repair system than does nuclear DNA.
2. Virtually all of the mitochondrial DNA strands encode important proteins, so that any defect will potentially affect mitochondrial function. Nuclear DNA contains long regions that do not encode proteins, wherein mutations or damage are essentially inconsequential.
3. Defective mitochondria often have a selection advantage over normal, active ones during proliferation and turnover.
4. Mitochondrial DNA is not protected by histones

Empirically, mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in cells subjected to oxidative stress or cancer chemotherapy agents like cisplatin due to both greater vulnerability and less efficient repair of mitochondrial DNA. Although mitochondrial DNA may be more sensitive to damage than nuclear DNA, it is relatively resistant, in some situations, to mutagenesis by chemical carcinogens. This is because mitochondria respond to some types of mitochondrial DNA damage by destroying their defective genomes rather than attempting to repair them. This results in global mitochondrial dysfunction for a period after cytotoxic chemotherapy. Clinical use of chemotherapy agents like cisplatin, mitomycin, and cytoxan is often accompanied by debilitating "chemotherapy fatigue", prolonged periods of weakness and exercise intolerance which may persist even after recovery from hematologic and gastrointestinal toxicities of such agents.

Compounds, compositions, and methods of the invention are useful for treatment and prevention of side effects of cancer chemotherapy related to mitochondrial dysfunction. This use of pyrimidine nucleotide precursors for attenuation of cancer chemotherapy side effects is mechanistically and biochemically distinct from toxicity reduction of cytotoxic anticancer - pyrimidine analogs by pyrimidine nucleotide precursors, which is mediated through biochemical competition at the level of nucleotide antimetabolites.

Example 5 illustrates the protective effect of oral triacetyluridine in protecting against taxol-induced neuropathy.

Furthermore, hepatic mitochondrial redox state is one contributor to appetite regulation. Cancer patients often display "early satiety", contributing to anorexia, weight loss, and cachexia. Energy metabolism is often seriously disrupted in cancer patients, with energy-wasting futile cycles of hyperactive tumor glycolysis producing circulating lactate, which is converted by the liver back to glucose. Chemotherapy-induced mitochondrial injury further contributes to metabolic disruption.

As indicated in Example 2, treatment with a compound of the invention resulted in improved appetite in a patient with mitochondrial disease.

*Mitochondria and ovarian function*

A crucial function of the ovary is to maintain integrity of the mitochondrial genome in oocytes, since mitochondria passed onto a fetus are all derived from those present in oocytes at the time of conception. Deletions in mitochondrial DNA become detectable around the age of menopause, and are also associated with abnormal menstrual cycles. Since cells cannot directly detect and respond to defects in mitochondrial DNA, but can only detect secondary effects that affect the cytoplasm, like impaired respiration, redox status, or deficits in pyrimidine synthesis, such products of mitochondrial function participate as a signal for oocyte selection and follicular atresia, ultimately triggering menopause when maintenance of mitochondrial genomic fidelity and functional activity can no longer be guaranteed. This is analogous to apoptosis in cells with DNA damage, which undergo an active process of cellular suicide when genomic fidelity can no longer be achieved by repair processes. Women with mitochondrial cytopathies affecting the gonads often undergo premature menopause or display primary cycling abnormalities. Cytotoxic cancer chemotherapy often induces premature menopause, with a consequent increased risk of osteoporosis. Chemotherapy-induced amenorrhea is generally due to primary ovarian failure. The incidence of chemotherapy-induced amenorrhea increases as a function of age in premenopausal women receiving chemotherapy, pointing toward mitochondrial involvement. Inhibitors of mitochondrial respiration or protein synthesis inhibit hormone-induced ovulation, and furthermore inhibit production of ovarian steroid hormones in response to pituitary gonadotropins. Women with

Downs syndrome typically undergo menopause prematurely, and also are subject to early onset of Alzheimer-like dementia. Low activity of cytochrome oxidase is consistently found in tissues of Downs patients and in late-onset Alzheimer's Disease.

Appropriate support of mitochondrial function or compensation for mitochondrial dysfunction therefore is useful for protecting against age-related or chemotherapy-induced menopause or irregularities of menstrual cycling or ovulation. Compounds and compositions of the invention, including also antioxidants and mitochondrial cofactors, are useful for treating and preventing amenorrhea, irregular ovulation, menopause, or secondary consequences of menopause.

In Example 1, treatment with a compound of the invention resulted in shortening of the menstrual cycle. Since the patient was in a persistent luteal phase, her response indicates that the administered pyrimidine nucleotide precursor reversed hyporesponsiveness to pituitary gonadotropins, which were presumably elevated to compensate for the ovarian hyporesponsiveness of mitochondrial origin.

#### *Diagnosis of mitochondrial disease*

The striking response of patients with mitochondrial disease to administration of compounds of the invention indicates that a clinical response to a pyrimidine nucleotide precursor administered according to the methods of the subject invention has diagnostic utility to detect possible mitochondrial disease. Molecular diagnosis of molecular lesions underlying mitochondrial dysfunction is difficult and costly, especially when the defect is not one of the more common mutations or deletions of mitochondrial DNA. Definitive diagnosis of mitochondrial disease often requires muscle biopsies, but even this invasive measure only works if mitochondrial defects are present in muscle. Since the compounds and compositions of the invention are safe when administered in accord with the methods of the subject invention, therapeutic challenge with a pyrimidine nucleotide precursor is an important diagnostic probe for suspected mitochondrial disease, especially when used in conjunction with tests for various other aspects of mitochondrial dysfunction.

For diagnosis of congenital mitochondrial cytopathy, daily doses of 50 to 300 mg/kg of a pyrimidine nucleotide precursor of the invention are administered to a patient for a period of one to twelve weeks and clinical signs and symptoms are monitored for changes. Improvements observed in the patients described in the Examples and additional patients include but are not limited to reduction of frequency and severity of seizures, migraines, and stroke-like episodes, improvement of weight gain in children with "failure to thrive", amelioration of renal tubular acidosis with concurrent reduction in the need for supplementary bicarbonate, improvement of muscular strength, improvement of speech acquisition, improvement of ataxia, improvement of hypotonia, reduction of the frequency and severity of sinus and ear infections, improvement of memory, and amelioration of symptoms of autonomic and peripheral neuropathy. In one embodiment of the invention, other tests of mitochondrial function are also used to provide evidence for diagnosis of mitochondrial disease. Diagnosis typically requires cumulative consideration of a number of corroborative tests with differing degrees of reliability, as described in Walker et al, (Eur Neurol., 36:260-7, 1996). Therapeutic responsiveness to a pyrimidine nucleotide precursor of the invention is primarily useful as an additional minor criterion in this diagnostic scheme, since it is possible that therapeutic benefits may occur after administration of pyrimidine nucleotide precursors that are not mediated solely by compensation for respiratory chain deficits. Since the nature and severity of symptoms of mitochondrial diseases are heterogeneous and variable between patients, efficacy of exogenous pyrimidine nucleotide precursors is typically assessed by selecting dominant symptoms in a patient and monitoring their severity with a quantitative scale as is feasible during a course of therapy. If a possible placebo effect is suspected, blinded switching of the patient from drug to an appropriate placebo is optionally used in an individual patient. Assessment of clinical benefit can require considerable skill and experience, but such skill is in the province of practitioners of the art of treating patients with multisystem metabolic diseases, and as such does not constitute undue experimentation, in view of the severity of this class of diseases. The examples cited below of clinical treatment of patients with mitochondrial diseases with triacetyluridine, a compound of the invention, exemplify the feasibility of determining clinical benefit in individual patients.

E. Administration and Formulation of Compounds and Compositions of the Invention

In the case of all of the specific therapeutic targets for pyrimidine nucleotide precursor therapy of mitochondrial disease, compounds of the invention are typically administered one to three times per day. Acyl derivatives of uridine and cytidine are administered orally in doses of 10 to 500 mg/kg of body weight per day, with variations within this range depending on the amount required for optimal clinical benefit. Generally, optimum doses are between 50 and 300 mg/kg/day (advantageously 100 to 300 mg/kg/day), divided into two or three separate doses taken 6 to 12 hours apart. Uridine and cytidine are less efficiently absorbed than are acyl derivatives of these two nucleosides, so that higher doses are required for therapeutic benefit comparable to that achieved with acyl derivatives. Osmotic diarrhea limits the amount of uridine or cytidine (or other derivatives like cytidine diphosphocholine) that can be administered to a patient, so that in most cases acyl derivatives of cytidine and uridine are more effective than the parent compounds, with fewer side effects. Doses of cytidine and uridine used to accomplish the purposes of the invention range from 50 to 1000 mg/kg/day, advantageously 100 to 1000 mg/kg/day, depending on the balance between therapeutic efficacy and tolerability. Orotate or alcohol esters of orotate are administered orally in doses ranging from 20 to 200 mg/kg/day, again depending on the amount needed to achieve an optimal therapeutic effect in a particular disease state involving mitochondrial respiratory chain dysfunction. The dose of pyrimidine nucleotide precursor of the invention required for a particular disease or patient will also depend in part on the severity of the disease.

In any individual patient with a disease characterized or caused by mitochondrial dysfunction, an effective dose of a pyrimidine nucleotide precursor of the invention is typically determined empirically. In congenital mitochondrial diseases, also known as mitochondrial cytopathies or mitochondrial encephalomyopathies, the clinical presentation of signs and symptoms is generally heterogeneous and variable between patients. Clinical benefit following administration of a compound of the invention is determined by monitoring a set of symptoms and assessing their severity over time, e.g. at monthly intervals. Three to five dominant symptoms are selected for this purpose, and the degree of amelioration judged to constitute clinical benefit is often a matter of clinical judgment. In treatment of patients with complex metabolic disorders, such assessment does not constitute undue burden of experimentation,

especially given the severity (often life threatening) of mitochondrial cytopathies and the costly nature of their care. Compensation for mitochondrial or other metabolic defects as early as possible in the patients life can make a very large difference versus intervention after development of the brain and body achieves stasis after puberty. It is therefore worthwhile for considerable effort to be expended on diagnosis and treatment of complex metabolic diseases, especially in developing children. The examples cited below of clinical improvement following administration of a compound of the invention to patients with mitochondrial diseases demonstrate the feasibility and value of such treatment and assessment.

In the case of most diseases with less heterogeneity in clinical presentation than mitochondrial disease, there exist in the art appropriate validated assessment scales for determining efficacy of drug treatments. Prior to conducting clinical studies to determine the doses of pyrimidine nucleotide precursors of the invention for treatment of the disease conditions disclosed in the instant specification, appropriate doses for individual patients are determined by evaluating clinical response (including brain MRI images and other indices, e.g. biochemical measurements, that may not necessarily be clinically apparent simply by observation of the patient's symptoms) according to quantitative disease assessment scales. In all cases, the dominant symptoms of a particular disease state are monitored over time to determine whether an improvement of signs and symptoms or attenuation of clinical decline occurs, as is common in the art of medicine. Prior to dose determination in blinded clinical studies, the response of a given patient to a pyrimidine nucleotide precursor of the invention is be differentiated from a possible placebo effect simply by blinded switchover from drug to placebo for a period of several weeks.

In the case of patients unable to receive oral medications, compounds of the invention, especially uridine, cytidine, and orotate esters can be administered, as required, by prolonged intravenous infusion, delivering daily doses of 10 to 500 mg/kg/day.

The pharmacologically active compounds optionally are combined with suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds. These are administered as tablets, suspensions, solutions, dragees, capsules, or suppositories. The compositions are administered for example orally,

rectally, vaginally, or released through the buccal pouch of the mouth, and may be applied in solution form by injection, orally or by topical administration. The compositions may contain from about 0.1 to 99 percent, preferably from about 50 to 90 percent of the active compound(s), together with the excipient(s).

For parenteral administration by injection or intravenous infusion, the active compounds are suspended or dissolved in aqueous medium such as sterile water or saline solution. - Injectable solutions or suspensions optionally contain a surfactant agent such as polyoxyethylenesorbitan esters, sorbitan esters, polyoxyethylene ethers, or solubilizing agents like propylene glycol or ethanol. The solution typically contains 0.01 to 5% of the active compounds.

Suitable excipients include fillers such as sugars, for example lactose, sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch or potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxymethyl cellulose and/or polyvinyl pyrrolidone.

Auxiliaries include flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions are used, which optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl cellulose phthalate or hydroxypropylmethyl cellulose phthalate are used. Dyestuffs or pigments are optionally added to the tablets or dragee coatings, for example, for identification or in order to characterize different compound doses.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use

are obtained by combining the active compound(s) with solid excipients, option-ally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Other pharmaceutical preparations which are useful for oral delivery include push-fit capsules made of gelatin, as well as soft-sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules contain the active compound(s) in the form of granules which optionally are mixed with fillers such as lactose, binders such as starches and/or lubricants such as talc or magnesium stearate, and, optionally stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids such as fatty oils, liquid paraffin, or polyethylene glycols. In addition, stabilizers optionally are added.

Pharmaceutical preparations which are used rectally include, for example, suppositories which consist of a combination of active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. In addition, gelatin rectal capsules which consist of a - combination of the active compounds with a base are useful. Base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons. In another - embodiment of the invention, an enema formulation is used, which optionally contains viscosity-increasing excipients like methylcellulose, hydroxypropylmethylcellulose, carboxymethylcellulose, carbopol, glycerine polyacrylates, or other hydrogels.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water soluble form, for example, water soluble salts.

In addition, suspensions of the active compounds as appropriate in oily injection suspensions are administered. Suitable lipophilic solvents or vehicles include fatty oils, for - example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions optionally include sub-stances which increase the viscosity of the suspension which include, for example, sodium carboxymethyl cellulose, sorbitol and/or dextran. The suspension optionally contains stabilizers.

F. Synthesis of the Compounds of the Invention

Acyl derivatives of cytidine and uridine are synthesized typically by acylation methods involving reaction of acid chlorides or acid anhydrides with cytidine or uridine.

The synthesis of 2',3',5'-tri-O-pyruvyluridine is shown in Example 6.

\* \* \*

The following examples are illustrative, but not limiting of the methods and compositions of the present invention. Other suitable modifications and adaptations of a variety of conditions and parameters normally encountered in clinical therapy which are obvious to those skilled in the art are within the spirit and scope of this invention.

### Examples

Example 1: Treatment of a multisystem mitochondrial disorder with triacetyluridine

A 29 year old woman with a partial Complex I deficiency, and whose son was diagnosed with mitochondrial disease leading to stroke-like episodes, ataxia, and encephalopathy, presented with a multisystem mitochondrial disorder. Signs and symptoms included hemiplegic/aphasic migraines, grand-mal seizures, neurogenic bowel and bladder dysfunction, requiring catheterization approximately four times per day, dysphagia, autonomic and peripheral polyneuropathy producing painful paresthesias, tachycardia Bradycardia syndrome, and poor functional capacity with inability to climb a flight of stairs without stopping to rest, and declining cognitive performance with episodes of clouded sensorium and poor memory lasting hours to days.

After beginning treatment with 0.05 mg/kg/day of oral triacetyluridine, and for a duration of at least 6 months, this patient has not had seizures or migraines; her paresthesias related to peripheral neuropathy have resolved. She is able to void spontaneously on most days, requiring catheterization only once or twice per week. After 6 weeks of treatment with

triacetyluridine, this patient was able to walk a full mile, which she has been unable to do for the past two years because of inadequate functional capacity. Her episodes of bradycardia during sleep and tachycardia during exertion have reduced infrequency; prior to treatment, tachycardia with a heart rate greater than 140 bpm occurred upon simple rise to stand, and after 6 weeks of triacetyluridine, tachycardia occurred only on hills and stairs. Her sensorium has cleared and memory deficits have improved markedly.

During treatment, this patients' menstrual cycles shortened from 4 weeks to two weeks, and she displayed a persistent luteal phase as evaluated by estradiol, progesterone, FSH and LH measurements. After several months, her cycle normalized to 4 weeks.

This patient demonstrates important features of the subject invention, in that 1) the compound of the invention caused improvements in virtually all features of a complex multisystem disease related to mitochondrial dysfunction in a variety of tissues, and that 2) compounds of the invention are unexpectedly useful for treating disease conditions related to a partial Complex I deficiency, which affects a portion of the mitochondrial respiratory chain that is outside of the sequence of electron transfers directly involved in de novo pyrimidine biosynthesis.

The transient shortening of this patient's menstrual cycle is interpreted as an improvement of ovarian function caused by triacetyl uridine in the face of excessive hormonal stimulation by which the neuroendocrine system was attempting to compensate for ovarian dysfunction. Feedback between the ovaries and the hypothalamus led to gradual normalization of cycle time.

Example 2: Treatment of refractory epilepsy

An 11 year old boy had refractory epilepsy since age 4.5, apparently due to a multiple mitochondrial DNA deletion syndrome. In December 1997, his condition deteriorated, including two admissions to an intensive care unit for crescendo epilepsy. Even with aggressive regimens of standard anticonvulsive therapy, this patient was having 8 to 10 grand-mal seizures

per night, leaving him unable to attend school regularly or participate in sports activities. He also developed upper lip automaticity.

In the first three days after beginning treatment with oral triacetyluridine (initially at a dose of 0.05 g/kg/day, and incrementally increased to 0.1 and then 0.24 g/kg/day over the course of several weeks), there were no seizures, and involuntary lip movements ceased. There has subsequently been some recurrence of seizures especially during episodes of infection, though at a much lower frequency than prior to treatment with triacetyluridine. This patient has been able to return to school and resume active participation in sports. His appetite, cognitive function, and fine motor coordination have improved during therapy, resulting in improved academic performance and in outstanding performance in sports activities like baseball.

Example 3: Treatment of renal tubular acidosis

A 2 year-old girl, with Leigh's Syndrome (subacute necrotizing encephalopathy) associated with severe Complex I deficiency, displayed renal tubular acidosis requiring intravenous administration of 25 mEq per day of sodium bicarbonate. Within several hours after beginning intragastric treatment with triacetyluridine at 0.1 g/mg/day, her renal tubular acidosis resolved and supplementary bicarbonate was no longer required to normalize blood pH. Triacetyluridine also resulted in rapid normalization of elevated circulating amino acid concentrations, and maintained lactic acid at low levels after withdrawal of dichloroacetate treatment, which was previously required to prevent lactic acidosis.

Example 4: Treatment of developmental delay

A 4.5 year-old girl with epilepsy, ataxia, language delay, and fat intolerance, and dicarboxylic aciduria was treated with triacetyluridine at a daily dose of 0.1 to 0.3 g/kg/day. Such treatment resulted in a 50% decline in seizure frequency, improvement of ataxia and motor coordination, restoration of dietary fat tolerance, and rapidly accelerated development of expressive language capabilities.

Example 5: Prevention of taxol-induced neuropathy

Peripheral neuropathy is a frequent, and often dose-limiting, side effect of important anticancer agents like cisplatin and taxol. In the case of taxol, sensory neuropathy occurs several days after administration. Taxol's mechanism of action involves stabilization of microtubules, which is useful for treating cancers, but is deleterious to peripheral neurons. - Microtubule stabilization impairs axonal transport of cellular components. Mitochondria shuttle between the cell body and terminals of neurons, so that the expression of mitochondrial - respiratory chain components can be regulated by nuclear transcription factors. During inhibition of mitochondrial shuttling, mitochondria distant from the nucleus undergo decline in expression of respiratory chain subunits encoded by the mitochondrial genome, due to inadequate exposure to mtDNA transcription factors, resulting in regional neuronal energy failure and other consequences of mitochondrial dysfunction.

Two groups of 10 mice each were treated with taxol, 21.6mg/kg/day for 6 consecutive days by intraperitoneal injection. An additional group of 10 mice received injections of vehicle alone. One of the groups of taxol-treated mice received oral triacetyluridine, 4000 mg/kg b.i.d. Nine days after the initiation of taxol treatments, nociceptive sensory deficits were tested by determining tail-flick latency after exposure of the tip of the tail to focused thermal radiation with an infrared heat lamp. In this system, delays in the tail-flick response to radiant heat correlate with sensory nerve deficits.

Group:	Tail flick latency
Control (no taxol)	10.8 ± 0.5 seconds
Taxol	16.0 ± 3.1 seconds
Taxol + triacetyluridine	11.9 ± 0.7 seconds

Taxol treatment impaired responses to painful stimuli as an index of toxic sensory neuropathy. Oral triacetyluridine treatment significantly attenuated taxol-induced alterations in tail-flick latency.

Example 6: Synthesis of Uridine Pyruvate

A. The preparation of pyruvyl chloride was accomplished by the reaction of alpha, alpha-dichloromethyl methyl ether and pyruvic acid using the procedure of Ottenheum and Man (Synthesis, 1975, p. 163).

B. Uridine (3.0 g, 12 mmol) was dried by toluene azeotrope undervacuum (3x), and then dissolved in DMF (20 mL) and pyridine (20mL). The resultant solution was cooled to -10 degrees C and 6.0mL of pyruvyl chloride (produced in step A above) was added dropwise. The reaction mixture was stirred at room temperature under argon for 24 hours. Analysis by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) showed the consumption of uridine. The reaction mixture was evaporated to dryness and partitioned between CH<sub>2</sub>Cl<sub>2</sub> and aqueous sodium bicarbonate. The organic layer was washed with water, aqueous HCl (pH 3.0), and water; dried over sodium sulfate; - concentrated; and purified using flash chromatography (silicagel, 5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) to yield 1.4 g of uridine pyruvate, or 2',3',5'-tri-O-pyruvyluridine.

Example 7: Therapeutic effect of oral triacetyluridine in the MPTP model of Parkinson's disease (PD) and mitochondrial dysfunction

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a complex I (NADH dehydrogenase) mitochondrial respiratory chain inhibitor that is used to induce dopaminergic cell loss (Varastet *et al.*, *Neuroscience*, 63: 47-56, 1994). This toxin is currently widely used as an animal model for PD (Bezard *et al.*, *Exp Neurol*, 148: 288-92, 1997).

Male C57/BL6 mice that were 6-9 months old weighing 30-40g from Taconic Farms were used in the MPTP studies (n=7/group). MPTP (30 mg/kg i.p.) was given b.i.d. for 1.5 days. TAU was administered b.i.d. 4g/kg p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL solution, 2 hours prior to toxin administration and until the day before sacrifice.

Eight days after stopping injection of MPTP, the mice were sacrificed by CO<sub>2</sub> and the striata from both sides were dissected out on cold surface. The striatum was frozen on dry ice. The dopaminergic neuronal survival was assessed by striatal dopamine (DA) content. The dopamine content was assayed by a radioenzymatic method under GLP conditions, but DA can also be measured using high pressure liquid chromatography with electrochemical detection as previously described (Friedemann & Gerhardt, *Neurobiol Aging*, 13: 325-32, 1992). There was a decreased mortality in the MPTP treated mice due to TAU treatment. The mortality in the control + MPTP mice was 71.4% compared to 28.6% in the TAU + MPTP treatment group. There was also a neuroprotective effect of PN401 treatment on the decrease in DA content due to MPTP.

#### Effect of TAU on MPTP-induced decrease in striatal DA content

Treatment	Striatal DA*
Control + Control	147 ± 13.0
TAU + Control	93.8 ± 10.7
Control + MPTP	9.2 ± 2.2
TAU + MPTP	37.9 ± 7.4

\* Data are represented as ng DA/mg protein (mean ± SEM).

A second study using MPTP (25 mg/kg i.p. b.i.d. for 2 days) was performed. Male C57/BL6 mice that were 6-9 months old weighing 30-40g from Taconic Farms were used in the MPTP studies (n=6/group). MPTP (30 mg/kg i.p.) was given b.i.d. for 2 days. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL solution 2 hours prior to toxin administration and until the day before sacrifice. TAU or vehicle was given orally (dose of TAU = 4g/kg bw b.i.d.) starting the day before MPTP administration and ending on day 8. Mice were sacrificed on day 9. This study also demonstrated that TAU showed protective effects on dopaminergic neurons as indicated by an attenuated decrease in striatal DA loss due to MPTP.

**Effect of TAU on MPTP-induced decrease in striatal DA content**

Treatment	Striatal DA*
Control + Control	71.0 ± 10.6
TAU + Control	52.0 ± 3.0
Control + MPTP	15.9 ± 2.2
TAU + MPTP	26.7 ± 0.9

\* Data are represented as ng DA/mg protein (mean ± SEM).

Example 8: Therapeutic effect of TAU in the 3-nitropropionic acid (3NP) model of Huntington's disease (HD)

HD is characterized by a progressive neuronal loss especially in the striatum. Patients with HD have a decreased activity of succinate dehydrogenase (complex II)- ubiquinol oxidoreductase (complex III) activity . Browne, Mitochondria & Free Radicals in Neurodegenerative Diseases, 361-380 (1997). A widely used model of HD employs an inhibitor of succinate dehydrogenase, 3-nitropropionic acid (3NP) . (Ferrante *et al.*, Mitochondria & Free Radicals in Neurodegenerative Diseases, 229-244, 1997). 3NP induces damage to the striatum in particular. (Brouillet *et al.*, J Neurochem, 60: 356-9, 1993).

Male 6-8 month old Swiss mice (National Cancer Institute; NCI, Frederick, MD) were treated with 3NP (65 mg/kg i.p.) daily for 4 days to induce mortality, neuronal cell loss and behavioral impairment with n=8/group. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL was given to the mice one day before and every day until day 8. On day 9, the mice were perfuse fixed with 10% buffered formalin and processed for silver staining to detect neuronal damage. There was decreased mortality due to 3NP in the mice treated with TAU compared to the controls as shown below.

There was no mortality in the 3NP + TAU group, but 3 of 8 mice died in the vehicle + 3NP group.

Behavioral scoring of the 3NP treated mice was to determine whether there was any motor impairment at anytime during the study. There were 88% of the control + 3NP treated mice with behavioral impairment indicated by gross observation. A decreased incidence of impairment of only 50% was found in the TAU + 3NP treated mice.

The silver staining was analyzed by a pathologist blinded to the identity of tissue samples. There were no clear signs of neuronal damage detected in the TAU + 3NP treated mice. However, in the control + 3NP treated mice, silver staining of synaptic terminals in the striatal area (caudate/putamen area) and substantia nigra was pronounced. Silver impregnation of axons and/or synaptic terminals in the thalamus, deep mesencephalon and/or reticular formation (medulla) was also found in 80% of the control + 3NP treated mice. The substantia nigra projects to the striatum and these areas are especially vulnerable to damage by 3NP. The damage to the substantia nigra and striatum was prevented by TAU.

Example 9: Therapeutic effect of TAU in the 3-nitropropionic acid (3NP) model of epilepsy

3-nitropropionic acid (3NP) is a mitochondrial toxin that acts by inhibiting Complex II of the respiratory chain; it is used to induce brain lesions similar to those characteristic of Huntington's disease. Seizures can also be induced by the use of 3NP as a model of epilepsy and mitochondrial dysfunction. Urbanska *et al.*, Eur J Pharmacol, 359: 55-8 (1998). Male CD-1 mice (National Cancer Institute, NCI, Frederick, MD) weighing between 26-40 g were used throughout. Mice were divided into groups of 5 and animals for each group were randomly chosen from different cages to avoid possible influence of age. The mice were maintained on a 12 hr light dark cycle with free access to water and food. All experiments were performed during the light period between 9:00 and 16:00 hr. Mice (n=17-20) were given 160 mg/kg 3NP and followed for seizures. 3NP was made up at 16mg or 18mg/ml in sterile water (pH: 7.4). 3NP was administered i.p. in a volume of 0.1ml/10g body weight. TAU was administered 4g/kg p.o. in 0.75% hydroxypropyl-methylcellulose vehicle 2 hours prior to 3NP administration. Seizures were assessed similar to the methods previously described (Roberts &

Keith, J Pharmacol Exp Ther, 270: 505-11, 1994; Urbanska *et al.*, Eur J Pharmacol, 359: 55-8, 1998).

Behavioral observations were performed within 120 min following application of 3-NP. Three major categories of convulsive seizure response were been considered and recorded:

1. Clonic movements: the movements of the forelimbs accompanied by facial twitching;
2. Explosive clonic movements: the movement of all four limbs involving running, jumping and bouncing;
3. Tonic response: including tonic flexion and tonic extension of the all four limbs.

Mortality rate was evaluated at 120 min after 3NP injection.

3NP induced primarily clonic seizures with some mice going on to develop a running and jumping behavior that generally resulted in mortality. TAU decreased the percent incidence of clonic seizure, running seizure and mortality due to 3NP. The primary endpoint was the latency to clonic seizure. TAU increased the latency to clonic seizure from 25.0-40.8 minutes. Data are represented as mean  $\pm$  SEM.

Endpoint	Control + 3NP	TAU + 3NP
% Clonic seizures	90.0	70.6
% Running seizures	42.9	5.9
% Mortality	35	11.8
Latency to clonic seizure	23.8 $\pm$ 0.7	40.8 $\pm$ 4.9

Example 10: Therapeutic effect of TAU in the quinolinic acid (OA) model of excitotoxicity

Quinolinic acid is an NMDA receptor agonist that has been used in models of Huntington's disease and excitotoxic damage (Beal *et al.*, J Neurosci, 11: 1649-59, 1991; Beal *et al.*, J Neurosci, 11: 147-58, 1991; Ferrante *et al.*, Exp Neurol, 119: 46-71, 1993). It can

induce severe damage to the CNS when administered directly into the striatum. The damage and/or mortality due to intrastriatal QA is likely due to a CNS etiology.

Male 6-8 month old Swiss mice (National Cancer Institute; NCI, Frederick, MD) were treated with QA (50 or 100 nmoles given bilaterally in both striatum n=8/group. TAU was administered b.i.d. 4g/kg bw p.o. in 0.75% hydroxypropyl-methylcellulose vehicle at 200 mg TAU /mL was given to the mice one day before and every day until day 6. On day 7 the mice were sacrificed. The QA was administered in a 2  $\mu$ l volume as previously described (Tatter *et al.*, *Neuroreport*, 6: 1125-9, 1995).

There was a decreased mortality due to QA in the TAU treated mice. The percent of mice surviving the 7 days treated with 50 nmoles QA was 64% in the control T QA and 73% in the TAU + QA and for mice treated with 100 nmoles QA only 4% survived in the control + QA group, whereas 19% survived in the TAU + QA group. TAU demonstrated a neuroprotective effect on the excitotoxicity due to QA.

\* \* \*

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations and modifications which come within the scope of the invention as claimed.

## CLAIMS

1. A method for treating or preventing pathophysiological consequences of mitochondrial respiratory chain dysfunction in a mammal comprising administering to said mammal in need of such treatment or prevention an effective amount of a pyrimidine nucleotide.
2. A method as in claim 1 wherein said respiratory chain dysfunction is caused by a mutation, deletion, or rearrangement of mitochondrial DNA.
3. A method as in claim 1 wherein said respiratory chain dysfunction is caused by defective nuclear-encoded protein components of the mitochondrial respiratory chain.
4. A method as in claim 1 wherein said respiratory chain dysfunction is caused by aging.
5. A method as in claim 1 wherein said respiratory chain dysfunction is caused by administration of cytotoxic cancer chemotherapy agents to said mammal.
6. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex I activity.
7. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex II activity.
8. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex III activity.
9. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex IV activity.
10. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex V activity.

11. A method as in claim 1 wherein said pyrimidine nucleotide precursor is selected from the group consisting of uridine, cytidine, an acyl derivative of uridine, an acyl derivative of cytidine, orotic acid, an alcohol ester of orotic acid, or a pharmaceutically acceptable salt thereof.
12. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of cytidine.
13. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of uridine.
14. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-tri-O-acetyluridine.
15. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-tri-O-pyruvyluridine.
16. A method as in claim 11 wherein the alcohol substituent of said alcohol ester of orotic acid is ethanol.
17. A method as in claim 11 wherein said pyrimidine nucleotide precursor is cytidine diphosphocholine.
18. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered orally.
19. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 10 to 1000 milligrams per kilogram of bodyweight per day.
20. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 100 to 300 milligrams per kilogram of bodyweight per day.

21. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a congenital mitochondrial disease.
22. A method as in claim 21 wherein said congenital mitochondrial disease is selected from the group consisting of MELAS, LHON, MERRF, MNGIE, NARP, PEO, Leigh's Disease, and Kearns-Sayres Syndrome.
23. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neurodegenerative disease.
24. A method as in claim 23 wherein said neurodegenerative disorder is Alzheimer's Disease.
25. A method as in claim 23 wherein said neurodegenerative disorder is Parkinson's disease.
26. A method as in claim 23 wherein said neurodegenerative disorder is Huntington's Disease.
27. A method as in claim 23 wherein said neurodegenerative disorder is age-related decline in cognitive function.
28. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neuromuscular degenerative disease.
29. A method as in claim 28 wherein said neuromuscular degenerative disease is selected from the group consisting of muscular dystrophy, myotonic dystrophy, chronic fatigue syndrome, and Friedreich's Ataxia.
30. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is developmental delay in cognitive, motor, language, executive function, or social skills.
31. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of epilepsy, peripheral -

neuropathy, optic neuropathy, autonomic neuropathy, neurogenic bowel dysfunction, sensorineural deafness, neurogenic bladder dysfunction, migraine, and ataxia.

32. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of renal tubular acidosis, dilating cardiomyopathy, steatohepatitis, hepatic failure, and lactic acidemia.
33. A method for preventing death or functional decline of post-mitotic cells in a mammal due to mitochondrial respiratory chain dysfunction comprising administration of an effective amount of a pyrimidine nucleotide precursor.
34. A method as in claim 33 wherein said post-mitotic cells are neurons.
35. A method as in claim 33 wherein said post-mitotic cells are skeletal muscle cells.
36. A method as in claim 33 wherein said post-mitotic cells are cardiomyocytes.
37. A method for treating developmental delay in cognitive, motor, language, executive function, or social skills in a mammal comprising administration of an effective amount of a pyrimidine nucleotide.
38. A method as in claim 37 wherein said developmental delay is pervasive developmental delay or PDD-NOS.
39. A method as in claim 37 wherein said developmental delay is Attention Deficit/Hyperactivity Disorder.
40. A method as in claim 37 wherein said developmental delay is Rett's Syndrome.
41. A method as in claim 37 wherein said developmental delay is autism.

42. A method for reducing side effects of cytotoxic cancer chemotherapy agents by administering a pyrimidine nucleotide precursor, where said cytotoxic chemotherapy agent is not a pyrimidine nucleoside analog.
43. A method as in claim 42 wherein said side effects of cytotoxic cancer chemotherapy are selected from the group consisting of peripheral neuropathy, chemotherapy-induced menopause, chemotherapy-associated fatigue, and depressed appetite.
44. A method for diagnosing mitochondrial disease by administering a pyrimidine nucleotide precursor and assessing clinical improvement in signs and symptoms in a mammal.
45. A compound selected from the group consisting of 2',3',5'-tri-O-pyruvyluridine, 2',3'-di-O-pyruvyluridine, 2',5'-di-O-pyruvyluridine, 3',5'-di-O-pyruvyluridine, 2'-O-pyruvyluridine, 3'-O-pyruvyluridine, and 5'-O-pyruvyluridine.
46. A pharmaceutical composition comprising:
  - (a) a pyrimidine nucleotide precursor or a pharmaceutically acceptable salt thereof, and
  - (b) pyruvic acid, a pharmaceutically acceptable salt thereof, or a pyruvic acid ester.
47. A method as in Claim 1 further comprising administering pyruvic acid, a pharmaceutically acceptable salt thereof, or a pyruvic acid ester.

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(54) **COMPOSITIONS AND METHODS FOR  
TREATING AND PREVENTING MEMORY  
IMPAIRMENT USING CITICOLINE**

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(57) **ABSTRACT**

This invention relates to compositions and methods for preventing and treating cognitive dysfunction or memory impairment. The compositions include an effective amount of citicoline, or pharmaceutically-acceptable salts thereof, and one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid. Other compositions of this invention include an effective amount of citicoline, or pharmaceutically-acceptable salt thereof, wherein said citicoline is metabolized to form at least one of cytidine, uridine, and choline. Still other compositions of this invention include effective amounts of choline, cytidine, and/or uridine, or their pharmaceutically-acceptable salts. This invention also encompasses methods for preparing these compositions.

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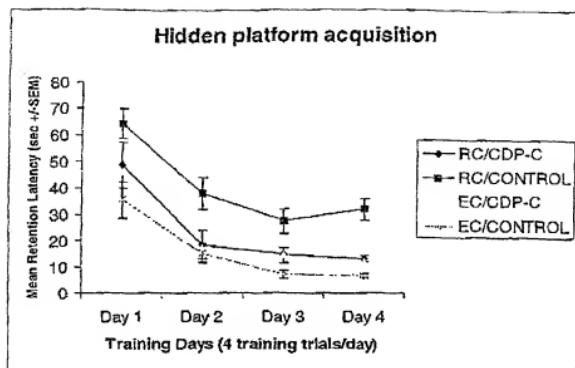


Fig. 1. Rats reared for 3 months in restricted conditions require more time than rats reared in enriched conditions to locate the hidden platform in this hippocampal-dependent memory task. A diet supplemented with CDP-choline improves this memory deficit in restricted rats.

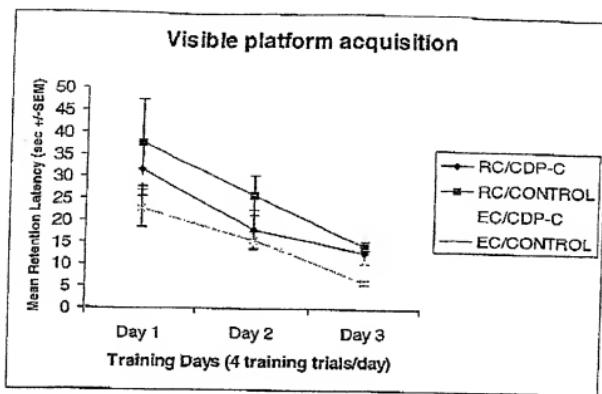


Fig. 2. Rats reared in restricted conditions do not require significantly longer retention times to locate the visible platform in this striatum-dependent memory task.

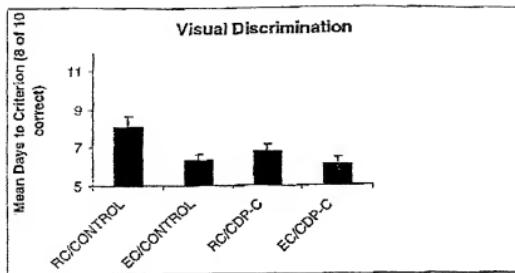


Fig. 3. Rats reared in restricted conditions acquire the simple visual discrimination task at a slower rate than do rats raised in enriched conditions. A diet high in CDP-choline alleviates some of this deficit.

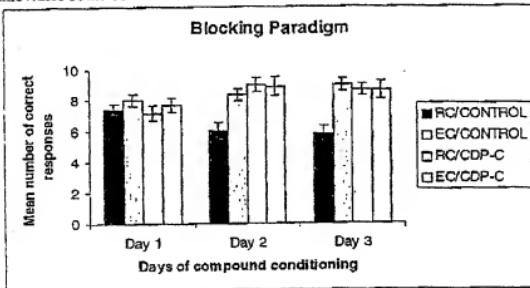


Fig. 4. Rats reared in restricted conditions are distracted by the addition of irrelevant cues to an already well-learned visual discrimination stimulus-response task. Enriched rats are not distracted by this irrelevant information attesting to their superior selective attention skills. CDP-choline alleviates this deficit in selective attention in restricted rats.

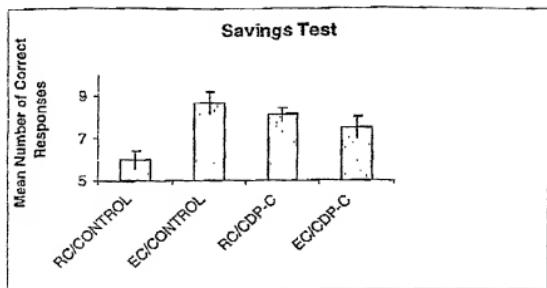


Fig. 5. A test for original savings of relevant information adds further evidence that original learning of the relevant information was impaired in restricted rats due to the addition of irrelevant information. A diet high in CDP-choline improves the ability of restricted rats to focus attention on the relevant information.

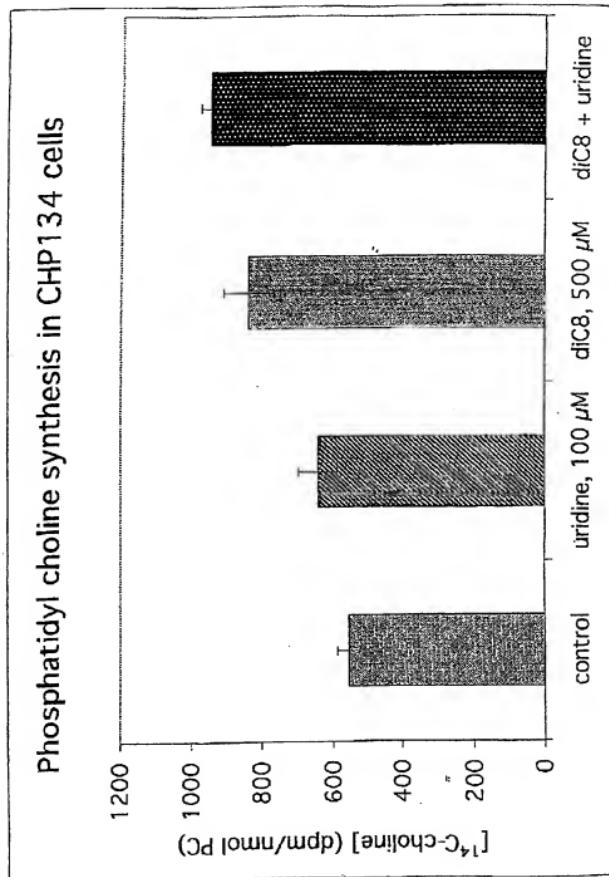


FIGURE 6

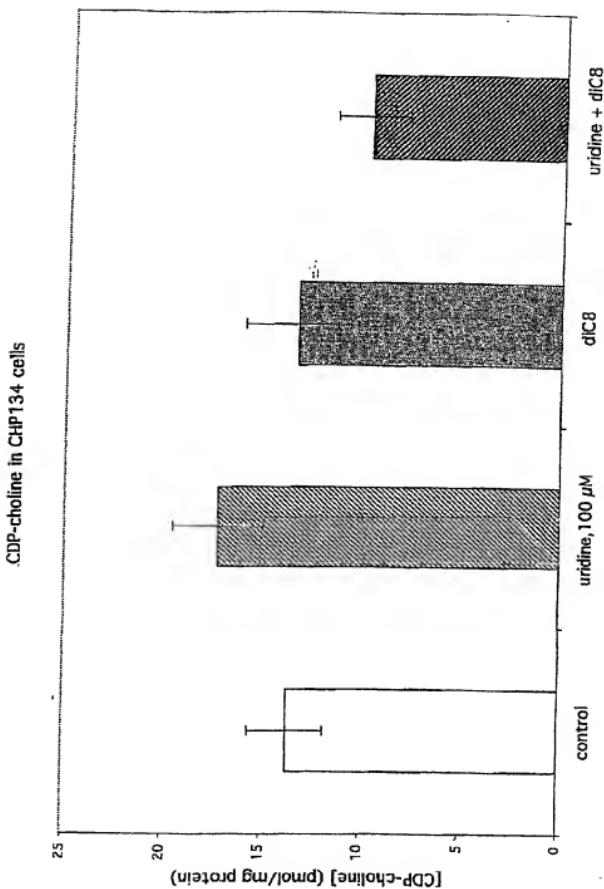


FIGURE 7

**COMPOSITIONS AND METHODS FOR TREATING  
AND PREVENTING MEMORY IMPAIRMENT  
USING CITICOLINE**

[0001] This application claims priority from U.S. Serial No. 60/339,445, filed Dec. 14, 2001.

**1. FIELD OF THE INVENTION**

[0002] This invention relates to compositions and methods for preventing and/or reducing memory impairment, particularly memory impairment caused by Mild Cognitive Impairment (MCI) or more severe dementias such as Alzheimer's Disease (AD), or other disorders including cerebrovascular disease. Administration of the compounds of this invention may enhance acetylcholine and membrane phosphatidyl choline synthesis, thereby increasing the amount of brain neurotransmitters available, and enhancing the growth of brain cells. More particularly, the invention relates to the use of compositions containing citicoline (cytidine-5'-diphosphocholine or CDP-choline) or its metabolites choline, cytidine, and/or uridine, and optionally one or more fatty acids, in a novel treatment regimen to prevent or reduce memory impairment and improve cognitive function and quality of life for patients suffering from these conditions.

**2. BACKGROUND OF THE INVENTION**

[0003] Memory impairment can be caused by a variety of disorders, and can range from mild to severe. The memory loss may be temporary or permanent, and may be the result of brain damage (either due to disease or trauma), depression, use of certain medications, emotional trauma, physiological changes in the brain, and reduction in the ability of the central nervous system to process information. Major theories held by neurologists believe that gradual deterioration of the brain causes memory loss. As people age, some neurons fail to function properly, or deteriorate to a non-functioning point, causing some problems with memory.

[0004] Age-Associated Memory Impairment (AAMI) refers to a decline in memory due to aging, characterized by temporary memory lapses in otherwise healthy individuals. Memory is commonly divided into two parts, short term and long term. Long term memory is affected less by aging than short term memory. Individuals with AAMI exhibit lapses in short term memory that are within the limits of what is considered "normal" for their age group. Such changes in memory in later life are often due to a combination of influences, including physiological changes in the brain, slowdowns in central nervous system processing abilities, certain diseases, and the use of certain medications (e.g., Aldomet (methyldopa), Ascendin (amoxapine), Dalmane (flurazepam), Elavil (amitriptyline), Equanil (mepramamate), Haldol (haloperidol), Inderal (propranolol), Mellaril (thioridazine), Miltown (meprothamate), Pamelor (nortriptyline), Pepcid (famotidine), Serax (oxazepam), Symmetrel (amantadine), Tagamet (cimetidine), Valium (diazepam), and Xanax (ramiprilene)). Other reasons may be reduced motivation to remember, or simple disease. It may be possible for individuals with AAMI to improve their memories by engaging in mental exercise, improving their diet, and incorporating exercise into their lifestyles.

[0005] Memory decline that is more severe or consistent than AAMI may be classified as Mild Cognitive Impairment

(MCI), and, in a minority of patients, indicates the early stages of a condition such as dementia. The memory lapses of MCI are more severe than age-related forgetfulness. In memory tests, people with MCI retain less information than most people their age. This memory impairment is also persistent, interfering with normal daily routines. According to studies, about 12 percent of people age 65 or older who were previously diagnosed with MCI are diagnosed with AD every year, making MCI one of the most important risk factors for AD.

[0006] The decline from MCI to Alzheimer's disease (AD) involves significant degradation of cognitive function, both in terms of the kinds of problems encountered and their severity. Patients with AD show abnormal memory impairment for their age group, as well as additional impairments in other mental skills. Loss of cholinergic neurons within the nucleus basalis has been correlated with cognitive impairment and disease severity through studies conducted using postmortem tissue from AD patients. AD is considered a form of dementia, which has symptoms including difficulties with language, learning, thinking and reasoning, as well as memory loss, and may also result in changes in mood and personality, eventually becoming severe enough to interfere with a person's work, everyday activities and social life. Other irreversible dementias include Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob disease and multi-infarct dementia. Some reversible causes of dementia include nutritional and vitamin deficiencies, drug intoxication, thyroid and blood chemistry imbalances, and tumors.

[0007] Cerebrovascular disease is also potentially devastating in terms of its effects on memory, and it is more common than AD. More than 700,000 Americans suffer a major cerebrovascular event—usually a stroke—each year. Stroke is the third leading cause of death in the United States and the number one cause of disability with more than 3,000,000 currently living with permanent brain damage, including memory loss, caused by such an event. The term cerebrovascular disease covers acute stroke and other diseases that may lead to stroke, like arteriovenous malformations (AVM), aneurysms, craniofacial venous malformations, brain tumors, spinal tumors, stenotic and thromboembolic occlusive diseases, and moyamoya. All of these cerebrovascular diseases have the potential to cause memory impairments, as well as other cognitive dysfunctions.

[0008] Various pharmaceutical therapies have been developed to combat memory loss, particularly memory loss associated with AD. These include:

[0009] Tacrine, or Cognex, which was the first drug approved in the US for the treatment of AD. It slows progression of AD by increasing levels of the neurotransmitter acetylcholine. It needs to be taken four times a day and blood tests for liver function need to be monitored. Up to six out of ten people are unable to reach the maximum dosage due to side effects.

[0010] Donepezil, or Aricept, is a widely known drug that was approved by the Food and Drug Administration (FDA) over four years ago for the treatment of memory loss related to AD. The drug raises the level of the chemical acetylcholine in the brain. By doing so, it slows decline of the disease. Recently, it has been used in other kinds of dementias as well as

reversible memory loss. There is growing literature about its use in various conditions including head trauma, mild cognitive impairment and other conditions. Side effects include gastrointestinal discomfort.

[0011] Rivastigmine, or Exelon, was approved by the FDA to treat AD. It also increases levels of acetylcholine in the brain. It is given twice a day and side effects include gastrointestinal discomfort.

[0012] Memantine, or Akaatinol, is an NMDA receptor agent that promotes nerve cell viability. It has been used in European countries including Germany to treat memory loss, but has not been approved for use in the US. Limited trials are ongoing at this time in the US.

[0013] Galantamine, or Reminyl, is an agent that raises brain levels of acetylcholine. Some preliminary data suggests that is more effective than earlier cholinergic agents. It is currently in limited US clinical trials and is not approved by the FDA. It has been in widespread use in Europe.

[0014] Neostigmine may promote the growth of nerve cells and maintain nerve cell viability. It is currently being used in trials abroad, but not in the US.

[0015] Nootropics, the first class of agents used for treatment of memory loss, have not been shown to be consistently effective and are not used routinely in the US.

[0016] Alpha-tocopherol, or vitamin E, in doses of 2000 IU has been shown to slow progression of Alzheimer's disease. The drug is believed to work as a free radical scavenger, and promote nerve cell viability.

[0017] Selegiline, or Eldepryl, is an agent that both raises the levels of certain neurochemicals and promotes nerve cell viability, and has been used in the US for the treatment of Parkinson's disease. It has been shown to be effective for the treatment of Alzheimer's.

[0018] Non-steroidal anti-inflammatory agents, or NSAIDS, include drugs such as ibuprofen (e.g., Motrin, Advil), as well as the newer cyclo-oxygenase 2 inhibitors (e.g., Celebrex and Vioxx). There is preliminary evidence to suggest that they may be helpful in treating some types of memory loss. Limited clinical trials are ongoing in the US.

[0019] Gingko Biloba, a free radical scavenger and possible brain activator, is commonly prescribed for the treatment of dementia in Europe. Preparations of the drug in the US vary, and the right dose of the right preparation may slow progression of some types of memory loss.

[0020] Estrogen therapy may help to prevent Alzheimer's disease in women.

[0021] B-secretase inhibitors are the newest class of drugs being developed for treating memory loss. These drugs stop formation of amyloid plaques, and

may halt the progression of illnesses like Alzheimer's. These drugs are currently involved in trials abroad.

[0022] Certain classes of the B vitamins (particularly B<sub>6</sub> and B<sub>12</sub>) are felt to be neuroprotective, and are being used in clinical trials for treating memory loss.

[0023] Calcium channel blockers, a class of drugs used to treat illnesses like hypertension and migraine, have been used to treat memory loss.

[0024] Citicoline monosodium is an exogenous form of cytidine-5'-diphosphocholine (CDP-choline). Endogenous CDP-Choline is a key intermediate in the biosynthesis of membrane phosphatidyl choline, which is of primary importance for the dynamic regulation of cellular integrity. The role of phospholipids in the maintenance of neuronal function is critically important in conditions such as MCI, AD, and various cerebrovascular diseases, where the breakdown of these membranes is thought to contribute to memory impairment. In addition to its role in neuron membrane structural function, phosphatidyl choline is thought to play a major role in lipid turnover (utilization of fatty acids) and communication signaling. It also acts as a neuroprotector. Citicoline donates the components choline, cytidine, and uridine (precursors to the synthesis of phosphatidylcholine), required to form, repair, and even restore function to nerve cell membranes. Cytidine and uridine, acting through cytidyl triphosphate (CTP), are also involved in the synthesis of other phospholipids. In addition, choline promotes the synthesis of acetylcholine, a neurotransmitter intimately associated with cognition. As an information-transmitting molecule, acetylcholine is necessary for proper memory function and is especially important for aging brains. In the brain, in addition to promoting phospholipid synthesis, citicoline also inhibits phospholipid degradation. Citicoline's mechanism of action is thought to entail cerebrovascular (blood circulation of the brain) regulation and neuroimmune (immune function of the nervous system) actions in the brain.

[0025] Citicoline has been extensively studied in clinical trials. Results of these trials indicated an improvement in a variety of clinical symptoms, including headache, vertigo, motor coordination and insomnia. These trials also showed improvements in motor function and reduction in stroke sequelae. However, such trials were limited to the use of citicoline during the rehabilitation stage of patients who may have suffered a stroke, and, thus, such treatments occurred well after the putative ischemic event. Nevertheless, such trials are informative for purposes of this invention because they show that stroke and head trauma patients tolerated citicoline well at dose ranges of 250 mg/day to 1000 mg/day for several weeks.

[0026] Although many pharmaceutical products have been developed in order to combat conditions that cause memory impairment, and particularly to slow or halt the progression of AD, none are highly effective in this regard. Further, none have been demonstrated to be effective in preventing the onset of symptoms of memory impairment. Accordingly, a need exists in the art for a composition and method for preventing or treating memory impairment.

### 3. SUMMARY OF THE INVENTION

[0027] The invention relates to compositions and methods for preventing and treating memory impairment, particularly

memory impairment caused by any of a number of disorders, such as stroke, brain injury, mild cognitive impairment (MCI), Alzheimer's Disease (AD), cerebrovascular disease, and other disorders which cause cognitive disturbances. The compositions according to this invention include citicoline, or its metabolism products, choline, cytidine, and/or uridine, and optionally one or more fatty acids such as linoleic acid and linolenic acid, and their active metabolites, e.g., arachidonic acid and docosahexenoic acid and any other essential fatty acids that are metabolized to form diacylglycerol (DAG). The methods include administering an effective amount of citicoline or a pharmaceutically-acceptable salt thereof, optionally in conjunction with these fatty acids, or administering any combination of choline, cytidine, and uridine, or the pharmaceutically-acceptable salts thereof, optionally in combination with these fatty acids.

[0028] The present invention also relates to the use of citicoline for the preparation of a pharmaceutical medicament for the prevention and treatment of memory impairment, comprising admixing an effective amount of citicoline, optionally with one or more fatty acids and a pharmaceutically acceptable carrier or as components of a food, or comprising admixing effective amounts of choline, cytidine, and/or uridine, optionally with one or more fatty acids and a pharmaceutically-acceptable carrier.

[0029] In the method of preventing and treating memory impairment, administration of an effective amount of citicoline preferably takes place over a specified period, typically over at least several weeks (e.g., 3-8 weeks, preferably at least about 6 weeks), and most preferably for an indefinite period. The dosage regimen can vary within certain limits. Typically, about 40-4000 mg of citicoline can be administered one or more times per day for the duration of the treatment period. The amount administered to a particular patient may vary based on several factors known to those of skill in the art, such as age, weight, severity of cognitive dysfunction, etc. The preferred dosage includes about 10-1000 mg of citicoline up to four (4) times a day, preferably about 2000 mg in a single dose or in divided doses. If choline, cytidine, and uridine are used, they are preferably provided in amounts commensurate with the amount of these compounds that are released when citicoline is metabolized. If fatty acids, such as linoleic acid and/or linolenic acid, are included, they are preferably provided in amounts thought to be sufficient to provide a beneficial amount of brain DAG.

[0030] Citicoline may be expected to have a number of advantages over other agents being developed for the prevention and treatment of memory impairment. Being an endogenous compound, citicoline is inherently safe. Citicoline has a very low toxicity and an extremely broad therapeutic index. The same applies to combinations of choline, cytidine, and uridine, as these are the natural by-products of the metabolism of citicoline in mammals. Fatty acids such as linoleic acid and linolenic acid are also low in toxicity, and are natural components of the diet of most animals.

[0031] The potential multimodal action of citicoline also may prove advantageous. Although the relative contribution of each potential mode of action to the treatment of cognitive dysfunction is not known, citicoline and its hydrolysis products—cytidine, uridine and choline—are believed to

play important roles in the generation of phospholipids involved in membrane formation and repair, as well as synthesis of acetylcholine, which is a brain neurotransmitter. These compounds also are believed to contribute to critical metabolic functions, such as the formation of nucleic acids and proteins. See, Ulus, I. H. et al. *Brain Research* (1989) 484:217-227. Thus, under conditions where memory impairment occurs, citicoline may function to (1) stabilize membranes by providing substrate for membrane maintenance; (2) repair damaged membranes by supplying important substrates for membrane formation; and (3) restore neuronal function by supplying substrate for the formation of acetylcholine. Moreover, unlike other proposed therapeutic agents, citicoline has the potential not only to prevent deterioration in cognitive ability, but also to contribute to the treatment of damaged areas of the brain, e.g., after stroke brain injury, thereby improving or restoring cognitive function.

[0032] It is, therefore, one object of the invention to provide a composition for preventing or treating memory impairment, comprising an effective amount of citicoline, or pharmaceutically-acceptable salt thereof, wherein said citicoline is metabolized to form at least one of cytidine, uridine, and choline.

[0033] Yet another object of the invention is to provide a composition for treating or preventing cognitive dysfunction, comprising an effective amount of citicoline, or pharmaceutically-acceptable salt thereof, and one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid or their active metabolites.

[0034] Still another object of this invention is to provide a composition for preventing or treating memory impairment, comprising at least one of an effective amount of choline or a pharmaceutically-acceptable salt thereof, an effective amount of cytidine or a pharmaceutically-acceptable salt thereof, and an effective amount of uridine or a pharmaceutically-acceptable salt thereof.

[0035] A further object of the invention is to provide a method for preparing a composition for use in treating or preventing memory impairment, comprising the steps of providing an effective amount of citicoline, or a pharmaceutically-acceptable salt thereof, providing an effective amount of one or more second compounds selected from the group consisting of linoleic acid and linolenic acid, and combining said citicoline with said one or more second compounds to form a pharmaceutically-acceptable preparation.

[0036] Another aspect of this invention is to provide a method for preparing a composition for use in treating of preventing memory impairment, comprising the steps of providing at least one of an effective amount of choline or a pharmaceutically-acceptable salt thereof, providing an effective amount of cytidine or a pharmaceutically-acceptable salt thereof, providing an effective amount of uridine or a pharmaceutically-acceptable salt thereof.

[0037] A further aspect of this invention is to provide a method of preventing or treating cognitive disorders, comprising the steps of administering at least one of an effective amount of choline or a pharmaceutically-acceptable salt thereof, administering an effective amount of cytidine or a pharmaceutically-acceptable salt thereof, administering an

effective amount of uridine or a pharmaceutically-acceptable salt thereof, and continuing to administer said citicoline for a period of at least about six weeks.

[0038] An additional object of this invention is to provide a method of preventing or treating memory impairment, comprising the steps of administering an effective amount of citicoline, or a pharmaceutically-acceptable salt thereof, combined with one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid, and continuing to administer said citicoline for a period of at least about six weeks.

[0039] These and other objects and aspects of the invention will be apparent to those of ordinary skill in view of the discussion above and the additional detailed description provided below relating to preferred embodiments of the invention.

#### 4. BRIEF DESCRIPTION OF THE DRAWINGS

[0040] FIG. 1 depicts a graph comparing the mean times taken by four groups of rats to acquire a hidden target. Rats were grouped according to whether they were reared in restricted or enriched conditions, and whether they received a diet supplemented with CDP-choline and unsaturated acids or a standard diet.

[0041] FIG. 2 depicts a graph comparing the mean times taken by the same four groups of rats to acquire a visible target.

[0042] FIG. 3 depicts a bar graph comparing the mean times taken by the same four groups of rats to acquire a visual discrimination task.

[0043] FIG. 4 depicts bar graphs comparing the mean correct responses given by the same four groups of rats when presented with distracting stimuli during a learned stimulus-response task.

[0044] FIG. 5 depicts a bar graph comparing the mean correct responses given by four groups of rats after originally learning relevant information while being presented with additional irrelevant information.

[0045] FIG. 6 depicts a bar graph comparing the amounts of phosphatidylcholine produced by CHP134 cells that were administered a choline-containing medium that also contains one of uridine, diacylglycerol, or a combination of uridine and diacylglycerol, as compared to control cells receiving only choline-containing medium.

[0046] FIG. 7 depicts a bar graph comparing the amounts of CDP-choline remaining in CHP134 cells that were administered a choline-containing medium that also contains one of uridine, diacylglycerol, or a combination of uridine and diacylglycerol, as compared to control cells receiving only choline-containing medium.

#### 5. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0047] This invention comprises compositions and methods for preventing and/or reducing memory impairment caused by MCI or AD, or by any of a number of other conditions, including those caused by cerebrovascular disease. The compounds of this invention include citicoline, and/or any combination of choline, cytidine, and uridine,

which are believed to increase production of membrane phosphatidyl choline and acetylcholine, thereby enhancing the growth of brain cells and increasing the availability of neurotransmitters. The compounds of this invention may also optionally include one or more fatty acids, such as linoleic acid or linolenic acid, or any other fatty acid that is metabolized to form diacylglycerol (DAG).

[0048] Without being limited by theory, it is believed that citicoline and its metabolites have at least a multiple mechanism of action: aiding in the repair of damaged neuronal tissues, enhancing the growth of axons and synapses, and aiding in the synthesis of brain neurotransmitter acetylcholine. Administration of citicoline is also believed to repair tissue damage by preventing the accumulation of potentially toxic free fatty acids which can be oxidized to generate free radicals, and the activation of enzymes that liberate these fatty acids. Upon oral or parenteral administration, CDP-choline releases its two principle components, cytidine and choline.

[0049] In humans, most of the cytidine is converted to uridine, which is phosphorylated to form uridine triphosphate (UTP), that is then converted to cytidine triphosphate (CTP). In addition, the choline is converted to its intermediate phosphocholine by the enzyme choline kinase. The CTP and phosphocholine together form CDP-choline in the brain, and the presence of DAG allows formation of phosphatidylcholine, a major neuronal membrane constituent. To promote availability of DAG, it is beneficial to administer a fatty acid such as linoleic acid or linolenic acid in conjunction with the citicoline, as these fatty acids are metabolized in part to form DAG. It is believed that DAG enhances the activity of choline phosphotransferase, although the invention is not bound to any particular theory. See Araki & Wurtman, Proc. Nat'l. Acad. Sci. U.S.A. 97: 11,946-11,950.

[0050] When administered orally, CDP-choline is absorbed almost completely, and its bioavailability is approximately the same as when administered intravenously. Once absorbed, it is metabolized to cytidine, uridine and choline which are dispersed widely throughout the organism, crossing the blood-brain barrier and reaching the central nervous system (CNS), where they are incorporated into the phospholipid fraction of the membrane and microsomes. CDP-choline activates the biosynthesis of structural phospholipids in the neuronal membranes, concurrently increasing cerebral metabolism and acting on the levels of various neurotransmitters. Due to these pharmacological activities, CDP-choline has a neuroprotective effect in situations of hypoxia and ischemia, and results in improved learning and memory performance in animal models of brain aging.

[0051] It is further postulated that to normalize brain function, nerve cells damaged by disorders affecting cognitive ability must manufacture new membrane elements, and the amount of neurotransmitters present in the brain must be increased. As described below, in preclinical animal models of cognitive impairment, administration of CDP-choline as a dietary supplement is shown to significantly improve cognitive function. It should be noted that the CDP-choline was also administered in conjunction with both linoleic acid and linolenic acid.

[0052] This invention is directed to a new and important use of citicoline and its metabolites: the treatment and

prevention of memory impairment caused by any of a number of underlying conditions. Although stabilization of membranes and increased production of acetylcholine are believed to be of benefit in treating cognitive dysfunction, it has not been definitively demonstrated that production of additional neurotransmitters and stabilization of membranes will improve cognitive function. The inventors have unexpectedly found that administration of citicoline significantly improves cognitive function, presumably by altering phosphatidylcholine synthesis and membrane formation and stimulating acetylcholine production, although this invention is not limited to any particular mechanism of action.

[0053] The citicoline-based compositions of this invention are generally preferred to be administered orally as a pharmaceutically-acceptable salt thereof. The preferred salt is the monosodium salt of citicoline, as this form is readily available in pharmaceutically-acceptable purity, although use of other pharmaceutically-acceptable citicoline salts is also envisioned. Further, where any combination of choline, cytidine, and uridine is used, it is also preferably administered orally as a pharmaceutically-acceptable salt or in a food.

[0054] Treatment under the invention is preferably begun prior to the onset of cognitive impairment symptoms, such as memory loss, e.g., in people who have had a stroke or brain injury, or shortly after such symptoms are first exhibited. However, the compositions and methods of this invention may also be beneficially administered to patients exhibiting advanced cognitive impairment in order to improve their cognitive function. In a specific embodiment of the invention, treatment is continued for at least up to about several weeks, preferably at least up to about several months, and most preferably for an indefinite period after the start of treatment.

[0055] Hence, according to one embodiment of the invention, a method is disclosed of treating memory impairment, and preventing future impairment, in a patient who has a disorder that causes cognitive dysfunction, comprising administering an effective amount of citicoline or a pharmaceutically acceptable salt thereof over a period of at least several weeks. Preferably, a fatty acid such as linoleic acid or linolenic acid is administered in conjunction with the citicoline. Preferably, the doses are administered at least once per day, and the treatment begins prior to the onset of symptoms of cognitive impairment. The preferred dose of about 40 to about 4000 mg of citicoline or its pharmaceutically acceptable salt may be administered one or more times daily. Alternatively, the method of this invention may comprise administering an effective amount of a combination of any of choline, cytidine, and uridine, or pharmaceutically-acceptable salts thereof, optionally in conjunction with administration of one or more fatty acids, over a period of at least several weeks.

[0056] The method of this invention finds its most advantageous use in human patients who have a disorder that results in memory impairment and other cognitive dysfunctions, including patients with MCI and AD. It cannot be stressed enough, however, that the citicoline, or the choline, cytidine, and uridine combinations, should be administered as soon as possible after symptoms of impairment begin to be exhibited. Further, citicoline, or the choline, cytidine, uridine combinations, may be administered as a preventative

measure to patients at risk for developing disorders that cause cognitive impairment. It is beneficial to co-administer these compositions with essential fatty acids that are metabolized to form DAG, such as linoleic acid or linolenic acid.

[0057] A variety of dosage ranges are suitable. The citicoline dosage under the invention may be from about 10 mg to about 1000 mg from one to about 4 times per day. For example, when a single daily dose is desired, citicoline is administered in from about 40 to about 4000 mg per day, preferably from about 500 to about 2000 mg per day. In one embodiment of the invention, the dose is 1000 mg per day. When choline and cytidine and/or uridine are used, the dose is preferably sufficient to provide these compounds in amounts that are commensurate with the amounts of choline, cytidine and/or uridine released when citicoline is metabolized.

[0058] For the composition of this invention, the amount of citicoline, or a pharmacologically-acceptable salt thereof required to achieve a therapeutic effect will vary with the route of administration and the particular disorder or disease to be treated. A suitable systemic dose of the active ingredient for a mammal suffering from, or likely to suffer from, any of the conditions described herein, is in the range of 40 mg to 4000 mg per day, with a preferred dose of 2000 mg per day. A dose of 1000 mg citicoline per day in a human will produce a plasma choline concentration of 1.5 ng/ml, the same as that produced by the administration of 500 mg/kg/day citicoline to the rat. It has been shown, however, that 500 mg per day conveys most of the benefits of citicoline treatment while minimizing any negative side effects, including dizziness, which may be experienced by some patients.

[0059] While it is possible for citicoline, or a combination of choline and cytidine and/or uridine to be administered alone, and in combination with fatty acids such as linoleic acid and linolenic acid, it may be preferable to present the active ingredients of the compositions of this invention as a formulation. Formulations of the active ingredients suitable for oral administration may be in the form of discrete units, such as capsules, cachets, tablets, or lozenges; in the form of a powder or granules for reconstitution; in the form of a solution or a suspension in an aqueous liquid or nonaqueous liquid; or, in the form of an oil-in-water emulsion or a water-in-oil emulsion or in a food base, e.g., pasta. The active ingredient also may be in the form of a bolus, electuary, or paste. Formulations of the active ingredient suitable for parenteral administration may comprise a sterile, aqueous preparation of the active ingredient. The formulations may be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacology.

[0060] In addition to containing the standard and well known pharmaceutical carriers and/or excipients, all of the above formulations may optionally contain one or more other therapeutically-active substances. Thus, this invention also contemplates a combination treatment regimen that relates to the administration of a composition including citicoline, or any combination of choline, cytidine, and uridine, with at least one additional therapeutic agent, or the respective pharmaceutically acceptable salts thereof.

[0061] Broad categories of the one or more optional additional therapeutic agents are contemplated. These agents

include, but are not limited to, "neuroprotectives" (e.g., inhibitors of the actions of excitatory amino acids, ACEA-1021, ACPC, Aptiganel, BW-619C, CNS-1145, CNS-1505, CPC-71 and CPC-702, Dextrophan and Dextromethorphan, Eliprodil, ES-242-1, FPL-15896, FR-115427, GP-1-4688, L-687414, L-689560, L-695902, LY-104658, LY-235959, LY-274614, LY-293558, Memantine, NNC-07-9202, NS-257, NPL-17742, "Protara", Remacemicid, Riluzole, SDZ EAA 494, Scifotol, SYM-1010, SYM-1207, YM-90K, MK-801; calcium channel blockers (e.g., AJ-394, AK-275, Calpain inhibitors, CD-349, Clentiazene, CNS-1237, CNS-2103, CPC-304 and CPC-317, Dazodipine, Diperidine, Emopamil, Fasudil, Lacidipine, Lifarizine, Lomerizine, Magnesium, MDL-28170, NB-818, Niyadipine, Nimo-dipine, NS-626 and related compounds, SM-6586, SNX-111, S-312-d, U-92022, UK-74506, US-035); agents targeted at nitric oxide; agents targeted at various other neurotransmitters (e.g., alpha<sub>1</sub>-receptor therapeutics, CV-5197, Dopamine receptors, Endolamine, Lazabemide, Milacipran, Nalmefene, RP-60180, SR-57746A, Synaptic uptake blockers); cytokines; hormones and related products (e.g., AN-100225 and AN-100226, Brain-derived neurotrophic factor, Calcitonin gene-related peptides, CEP075 and related compounds, Ciliary neurotrophic factor, Endothelial cell factor, Endothelin inhibitors, FR-139317 Interleukin-1 receptor antagonist (lipocortin), JTP-2942, Macrophage-regulating compounds, Motoneuronotrophic factor NBI-117, Nerve growth factor, Neural stem cells, Neutrophil inhibitor factor, NS-506, NT-3, Positacrine, Schwann cell promoters, sCR1, Somatomedin-1); free radical scavengers (e.g., EPC-K1, MCI-186, Nicaravirin, Phenazoxazin, Resatoran, Rumben, Superoxide dismutase, Tirilazad mesylate, U-88999E, Yissum project P-0619, YM-737); gangliosides and related products (e.g., LIGA4, LIGA4, Monosialoganglioside (GM1), ND-37, Siagosome).

[0062] Still other classes of the one or more optional additional therapeutic agents include, but are not limited to, modulators of various specific enzymes (e.g., CEP-217, CEP-245, CEP-592, CNS-1531, Ebselfen, Epafrestat, JTP-4819, K-7259, Protease nexin-1, SK-827, Tyrosine kinase modulators, Z-321); memory enhancers or "nootropics" (e.g., Aloracetam, Choline-L-aloscerate, DN-2574, Idebenone, Oxiracetam, Piracetam, Pramiracetam, Tacrine and its analogues, Vinconate); neuroprotectives with "diverse" actions (e.g., Ademetionine sulphate tosylate, Ancred, Apocuanzine, CPC-111, CPC-211, HSV vectors, KF-17329 and KF-19863, LY-178002, MS-153, Nicorandil, N-3393 and N-3398, SUN 4757, TJ-8007, VA-045); and imaging or contrast agents.

[0063] Therefore, methods and compositions are provided for treating a subject who is experiencing memory impairment or other forms of cognitive dysfunction, comprising administering a composition including an effective amount of citicoline, or effective amounts of choline, cytidine, and/or uridine, optionally including one or more fatty acids such as linoleic or linolenic acid that metabolize to form DAG, and at least one additional therapeutic agent, or their respective pharmaceutically acceptable salts, shortly after the onset of symptoms. More preferably, the composition is administered before symptom onset for patients at risk for developing cognitive disorders. The treatment regimen may be continued for a period of several weeks, several months, or indefinitely, depending on the condition of the patient.

[0064] The method of using the contemplated compositions includes the administration or co-administration of subsequent doses, which is preferably carried out over a period of at least about several weeks. In specific embodiment of the invention, the administration of the compositions of this invention is carried out over a period of at least about several weeks, preferably over a period of at least about several months, and most preferably over an indefinite period. Furthermore, the doses are administered one or more times daily over the treatment period. It is anticipated that subjects who may benefit the most from this therapy are those who suffer from MCI or AD.

[0065] Further, the compositions of this invention may also optionally include or be administered in conjunction with additional therapeutic agents, such as NSAIDs, ginkgo biloba, vitamin E, and certain B vitamins, which may be useful in treating or preventing memory impairment associated with MCI or AD.

[0066] Hence, a composition is likewise provided for the treatment of a subject experiencing memory impairment, comprising an effective amount of citicoline, or effective amounts of choline, cytidine, and/or uridine, optionally including one or more fatty acids that metabolize to form DAG, and one or more additional therapeutic agents, or their respective pharmaceutically-acceptable salts, in a pharmaceutically-acceptable carrier. In such a composition the effective amount of active ingredients may vary according to the particular needs of the patient. Typical ranges, however, may be from about 40 mg to about 4000 mg of citicoline and about 10 mg to about 500 mg of the one or more optional additional therapeutic agents.

[0067] The present invention is illustrated by the Examples that follow, it being understood, however, that the invention is not limited to the specific details of these Examples.

## 6. EXAMPLES

### [0068] 6.1. Animal Trial

[0069] The ability of citicoline to prevent or minimize the effects of memory impairment was demonstrated in a model of memory impairment in the rat. The impairments studied in rats are very similar to the type that occur in people with age-related memory impairment (or "minimal cognitive impairment"), as well as in more severe dementias, e.g., following stroke, brain injury or Alzheimer's Disease, in that they involve the same part of the brain, i.e., the hypothalamus. The memory impairment model in the rat was produced by separating rats weaned at 23 days old into four groups, and introducing them into either enriched or restricted environmental conditions, with or without supplementation of their diets with citicoline (500 mg/kg/day), linoleic acid, and linolenic acid for three months. Behavioral testing was conducted over a six week period during which the environmental and dietary conditions continued.

[0070] Animals from the group subjected to restricted environmental conditions that did not receive diets supplemented with citicoline exhibited a significant impairment in hippocampus-dependent types of memory. These animals were also susceptible to distraction by placing other objects in their environments. However, animals from the group subjected to restricted environmental conditions that did

receive diets supplemented with citicoline, linoleic acid, and linolenic acid were protected against developing these memory impairments, and performed in a manner consistent with that exhibited by the rats raised in enriched conditions. Addition of citicoline, linoleic acid, and linolenic acid to the diets of rats raised in enriched conditions did not appear to produce any effect on ability to learn tasks, or to perform them when presented with distracting stimuli.

[0071] Referring to FIG. 1, this graph shows the results of behavioral testing using a hippocampal-dependent memory task. Rats reared under restricted conditions that were not provided with a diet supplemented with citicoline, linoleic acid, and linolenic acid took longer to acquire a hidden platform than rats raised in enriched conditions or rats raised in restricted conditions that received a diet supplemented with citicoline, linoleic acid, linolenic acid. This study shows that the supplementation with citicoline, linoleic acid, and linolenic acid improves the memory deficit of rats raised under restricted conditions, to the point that they are able to perform the task with efficiency similar to that of rats raised under enriched conditions.

[0072] Referring to FIG. 2, when presented with a striatum-dependent memory task, rats reared in restricted conditions perform almost as well as rats raised in enriched conditions, regardless of whether they received a diet supplemented with citicoline, linoleic acid, and linolenic acid.

[0073] As shown in FIG. 3, rats reared in restricted conditions require a longer period of time to acquire a simple visual discrimination task than do rats raised in enriched conditions. Providing a diet supplemented with citicoline, linoleic acid, and linolenic acid alleviates some of the deficit.

[0074] FIG. 4 illustrates the effect of distraction by irrelevant cues while performing a well-learned visual discrimination stimulus-response task. Rats reared in restricted conditions were more distracted by the irrelevant information, while rats reared in enriched conditions were less distracted, evidencing their superior selective attention skills. Dietary supplementation with citicoline, linoleic acid, and linolenic acid alleviated the deficit in selective attention in rats reared in restricted conditions.

[0075] The test depicted in FIG. 5 compares the ability to learn and retain relevant information. Rats reared in restricted conditions were impaired in their ability to learn the relevant information because of the presence of additional irrelevant information. Rats reared in restricted conditions that received a diet supplemented with citicoline, linoleic acid, and linoleic acid were better able to focus attention on the relevant information.

#### [0076] 6.2 Cell Cultures

[0077] The ability of diacylglycerol (DAG, or diC8 as shown in FIGS. 6 and 7) to enhance the synthesis of phosphatidylcholine was assessed in cell cultures of the human-derived CHP 134 cell line. The cell cultures were exposed to medium containing choline, and the cell cultures were then divided into four groups according to whether no additional compounds were added to the medium (control group), 100  $\mu$ M uridine was added to the medium (uridine group), 500  $\mu$ M diacylglycerol was added to the medium,

(diC8 group), or 100  $\mu$ M uridine and 500  $\mu$ M diacylglycerol were added to the medium (uridine+diC8 group).

[0078] FIG. 6 compares the amount of phosphatidylcholine formed by each of the four groups of cell lines. The results indicate that the cell culture exposed to choline, uridine, and DAG produced the highest levels of phosphatidylcholine, followed by the cell culture exposed to choline and DAG. The control cell culture showed the lowest level of phosphatidylcholine production. These results highlight the usefulness of providing a source of DAG along with choline and uridine.

[0079] FIG. 7 compares the amount of CDP-choline remaining in each of the four groups of cell lines, with lower levels indicating that more CDP-choline was converted to phosphatidylcholine. The results indicate that the cell culture exposed to choline, uridine, and DAG had the lowest levels of CDP-choline remaining, which is consistent with the results obtained in FIG. 6 that indicated that this group produced higher levels of phosphatidylcholine. The cell culture supplemented with choline and DAG exhibited lower levels of CDP-choline than did the group supplemented with choline and uridine. The control group contained CDP-choline at a level commensurate with that of the choline and DAG group. This result is not unexpected due to the role of DAG in combining with CDP-choline to form phosphatidylcholine. The absence of additional DAG in the uridine and choline-supplemented group likely resulted in increased production of CDP-choline, without enough DAG to allow all of it to be converted to phosphatidylcholine.

[0080] Other embodiments should be apparent to those of ordinary skill in view of the detailed disclosure provided herein, which embodiments would nonetheless fall within the scope and spirit of the present invention. Hence, the preceding preferred embodiments should not be construed as limiting the invention in any way.

#### What is claimed is:

1. A composition comprising citicoline, or a pharmaceutically-acceptable salt thereof, wherein said citicoline is metabolized to form at least one of cytidine, uridine, and choline.
2. The composition of claim 1, further comprising one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid or their active metabolites.
3. The composition of claim 2, wherein the active metabolite is arachidonic acid or docosahexanoic acid.
4. A composition comprising at least one of choline or a pharmaceutically-acceptable salt thereof, cytidine or a pharmaceutically-acceptable salt thereof, and uridine or a pharmaceutically-acceptable salt thereof.
5. The composition of claim 4, further comprising a fatty acid.
6. The composition of claim 4, wherein the fatty acid is selected from the group consisting of linoleic acid and linolenic acid or their active metabolites.
7. A method for preparing a composition for use in treating or preventing memory impairment or cognitive dysfunction, comprising the steps of:
  - providing an effective amount of citicoline, or a pharmaceutically-acceptable salt thereof;

providing an effective amount of one or more second compounds selected from the group consisting of linoleic acid and linolenic acid; and

combining said citicoline with said one or more second compounds to form a pharmaceutically-acceptable preparation.

8. A method for preparing a composition for use in treating or preventing memory impairment or cognitive dysfunction, comprising the steps of:

providing an effective amount of choline or a pharmaceutically-acceptable salt thereof;

providing an effective amount of cytidine or a pharmaceutically-acceptable salt thereof; and

providing an effective amount of uridine or a pharmaceutically-acceptable salt thereof.

9. A method of preventing or treating cognitive disorders or memory impairment, comprising the steps of:

administering an effective amount of choline or a pharmaceutically-acceptable salt thereof;

administering an effective amount of cytidine or a pharmaceutically-acceptable salt thereof;

administering an effective amount of uridine or a pharmaceutically-acceptable salt thereof; and

continuing said administration for a period of at least about six weeks.

10. The method of claim 9, further comprising the step of administering one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid.

11. A method of preventing or treating cognitive disorders or memory impairment, comprising the steps of:

administering an effective amount of choline or a pharmaceutically-acceptable salt thereof;

administering an effective amount of cytidine or a pharmaceutically-acceptable salt thereof; and

continuing said administration for a period of at least about six weeks.

12. The method of claim 11, further comprising the step of administering one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid.

13. A method of preventing or treating cognitive disorders or memory impairment, comprising the steps of:

administering an effective amount of choline or a pharmaceutically-acceptable salt thereof;

administering an effective amount of uridine or a pharmaceutically-acceptable salt thereof; and

continuing said administration for a period of at least about six weeks.

14. The method of claim 13, further comprising the step of administering one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid.

15. A method of preventing or treating cognitive disorders or memory impairment, comprising the steps of:

administering an effective amount of cytidine or a pharmaceutically-acceptable salt thereof;

administering an effective amount of uridine or a pharmaceutically-acceptable salt thereof; and

continuing said administration for a period of at least about six weeks.

16. The method of claim 15, further comprising the step of administering one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid.

17. A method of preventing or treating cognitive disorders or memory impairment, comprising the steps of:

administering an effective amount of citicoline, or a pharmaceutically-acceptable salt thereof, combined with one or more of the compounds selected from the group consisting of linoleic acid and linolenic acid; and

continuing to administer said citicoline for a period of at least about six weeks.

\* \* \* \* \*

P. Yamamoto et al. (U.S. Patent No. 5,635,486) – entered September 12, 2005



US005635486A

**United States Patent** [19]

Yamamoto et al.

[11] **Patent Number:** 5,635,486[45] **Date of Patent:** Jun. 3, 1997[54] **OPHTHALMIC COMPOSITION  
COMPRISING A SLEEP ADJUSTING  
SUBSTANCE**[75] **Inventors:** Keizo Yamamoto, Takasago; Takayoshi Hidaka, Kobe, both of Japan[73] **Assignee:** Kanegafuchi Kagaku Kogyo Kabushiki Kaisha, Osaka, Japan[21] **Appl. No.:** 302,236[22] **Filed:** Sep. 7, 1994**Related U.S. Application Data**

[63] Continuation of Ser. No. 993,421, Dec. 21, 1992, abandoned.

[30] **Foreign Application Priority Data**

May 11, 1990 [JP] Japan 2-121786

[51] **Int. Cl. 6** A61K 31/70; A61K 31/495[52] **U.S. Cl.** 514/32; 514/255; 514/912[58] **Field of Search** 514/32, 255, 912[56] **References Cited****PUBLICATIONS**

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Attorney, Agent, or Firm—Reiner, Otto, Boisselle & Sklar**

[57] **ABSTRACT**

The present invention provides an ophthalmic composition comprising a sleep inducing substance or a sleep inhibiting substance, and the sleep inducing substance or the sleep inhibiting substance is derived from an organism. Thus, the invention provides an ophthalmic composition comprising a sleep adjusting substance which normalizes irregularity of the rhythm of sleep and a liquid ophthalmic medicament comprising the composition, the composition and the medicament being safe and obtainable by anyone, and a method for regulating the rhythm of sleep comprising instilling the ophthalmic medicament including the sleep adjusting substance to an individual.

**8 Claims, 3 Drawing Sheets**

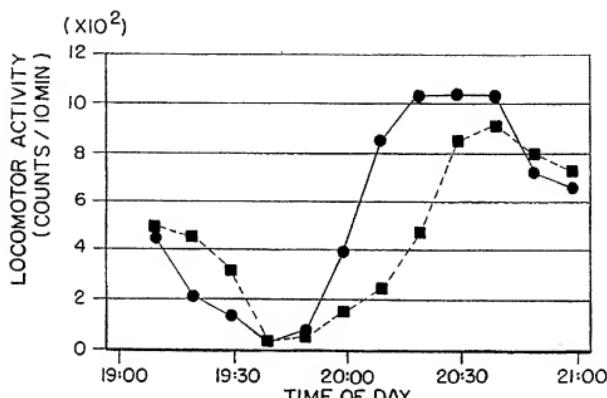


FIG. 1

—●— SALINE  
-■- URIDINE (100 $\mu$ M)

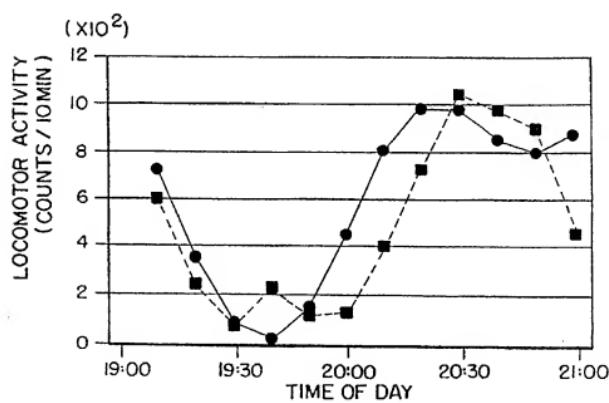


FIG. 2

—●— SALINE  
-■- PGD<sub>2</sub> (100 $\mu$ M)

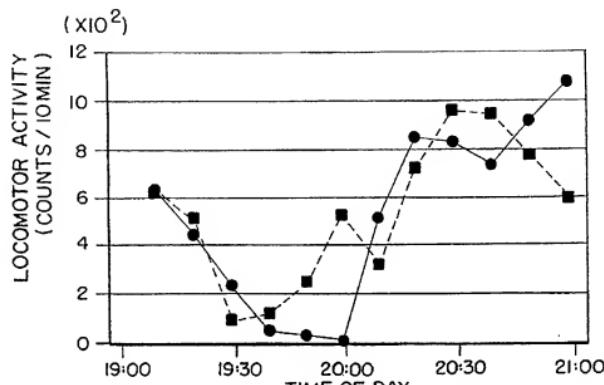


FIG. 3

● SALINE  
- - CYTIDINE (100  $\mu$ M)

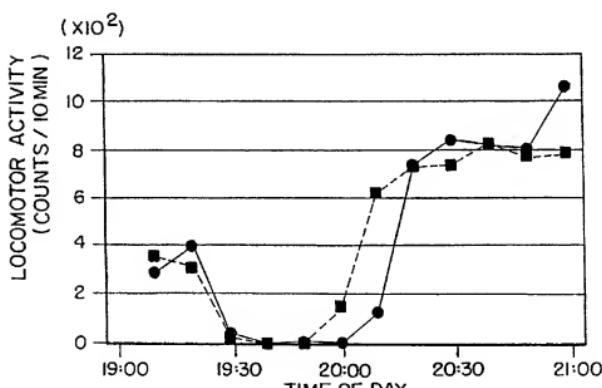


FIG. 4

● SALINE  
- - DSIP (100  $\mu$ M)

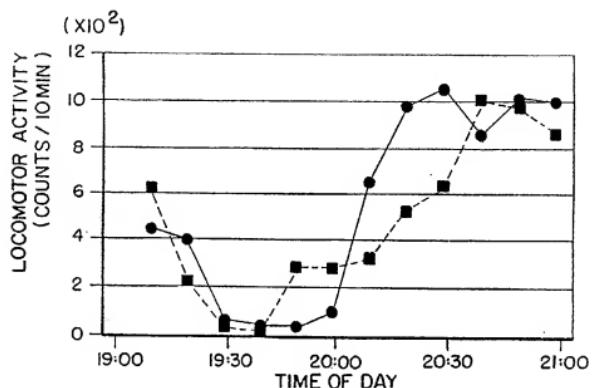


FIG. 5

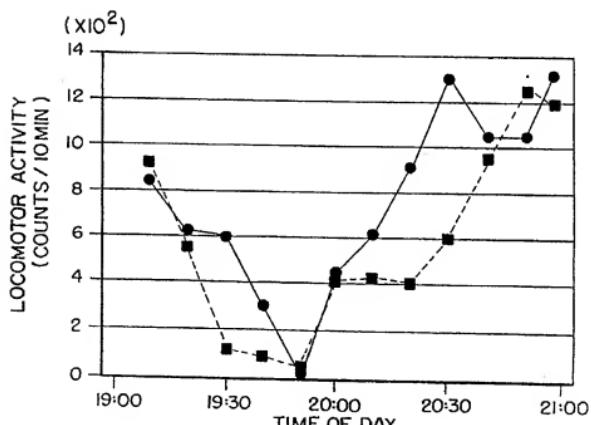


FIG. 6

OPHTHALMIC COMPOSITION  
COMPRISING A SLEEP ADJUSTING  
SUBSTANCE

This is a continuation application Ser. No. 07/993,421 filed on Dec. 21, 1992, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to an ophthalmic composition comprising a substance for regulating the rhythm of sleep, and a method for regulating the rhythm of sleep by the instillation of a medicament comprising the composition to the eye.

2. Description of the Related Art

Synthetic sleeping drugs have been used to correct the disorder in the rhythm of sleep. A synthetic sleeping drug is administered, if necessary, for example, to those suffered from a so-called jet lag after an overseas trip, a deteriorated physical condition due to three-shift work, nycteric poromania due to senile dementia, and insomnia due to stress. However, since an excessive dose of some of the synthetic sleeping drugs can cause the death of a taker, close attention must be paid to their administration.

Such synthetic sleeping drugs are divided into two types: a barbiturate type and a non-barbiturate type. The barbiturate type sleeping drugs have a strong effect and are highly dangerous. The non-barbiturate type sleeping drugs have relatively moderate effects.

Among such non-barbiturate type sleeping drugs, benzodiazepine type sleeping drugs such as Halcion (trade name) are regarded to be comparatively safe and are widely utilized clinically these days. But it is known that these drugs can cause anterograde amnesia. It is unknown how the amnesia occurs, but the cause is presumed to be as follows. Sleep is generally divided into two types on the basis of the characteristics thereof: Non-REM sleep (slow wave sleep) and REM sleep (paradoxical sleep). The physiological meanings of the two types have not been fully understood as yet, but it is known that a rat that has been experimentally deprived of REM sleep, that is, a REM sleep deprivation rat, suffers from memory defect for a long period of time. Therefore, REM sleep is definitely concerned with memory fixing. The benzodiazepine type sleeping drugs lower the function of the brain and shorten the duration of REM sleep, and so they can induce anterograde amnesia. Therefore, such benzodiazepine type sleeping drugs are not suitable for patients with senile dementia who can easily fall into amnesia or other defects of memory and for students preparing themselves for an examination who should maintain their memory as clearly as possible.

Since the synthetic sleeping drugs are somewhat dangerous in this manner, it is impossible to obtain them without the prescription of a doctor, which causes inconvenience for those who are not under medical treatment.

On the other hand, sleep inducing substances derived from an organism have been reported as being different from the above described synthetic sleeping drugs. Substances derived from an organism herein mean substances existing in a body of an organism. Examples of such substances include those synthesized in the body of an organism and those from outside a body and utilized after intake for metabolism. It is known that these sleep inducing substances induce natural and physiological sleep without reducing the hours of REM sleep (Borbely et al., *Physiol. Rev.*, 69: 605

(1989)). Such substances do not cause anterograde amnesia as the synthetic sleeping drugs do. Sleep inhibiting substances derived from an organism are also known, and there is no report of any habituation by using such sleep inhibiting substances over a long period of time.

Therefore, these sleep inducing substances and sleep inhibiting substances both derived from organisms (hereinafter referred to as the "sleep adjusting substances") are very useful in treating any disorder in the rhythm of sleep.

10 As an administration method of a sleeping drug, an oral administration or an injection is generally used, and the oral administration is preferred because it is simple. However, the sleep inducing or sleep inhibiting substances derived from organisms is significantly effective only when they are administered directly into a cerebral ventricle. There are a few reports that it was effective to administer them into an abdominal cavity. But the administration into the cerebral ventricle or abdominal cavity is neither convenient nor practical. As the oral administration of these substances, relief of jet lag by orally administering melatonin has been reported (Arndt et al., *Br. Med. J.*, 292: 1170 (1986); and oral administration of tryptophan is known. In these cases, the oral administration is somewhat effective, but there is no report, except for the above, that the oral administration of the above described sleep adjusting substances derived from an organism is effective. This is because these substances have a disadvantage that they are easily metabolized in an organism and scarcely reach the region for adjusting the biological rhythm in the brain since they are derived from an organism. Therefore, it has been impossible to put these substances to practical use.

SUMMARY OF THE INVENTION

The ophthalmic composition of the invention comprises 35 at least one sleep adjusting substance.

In one aspect of the invention, the sleep adjusting substance is derived from an organism.

In another aspect of the invention, the sleep adjusting substance derived from an organism is at least one sleep inducing substance selected from the group consisting of uridine, sleep promoting substance, adenosine, prostaglandin D<sub>2</sub>, delta-sleep-inducing peptide, piperidine, 2-octyl-gamma-bromoacetate, arginine vasotocin, melatonin, serotonin, tryptophan, oxidized glutathione, and derivatives 45 thereof.

In another aspect of the invention, the sleep adjusting substance derived from an organism is at least one sleep inhibiting substance selected from the group consisting of cytidine, prostaglandin E<sub>2</sub>, delta-sleep-inducing peptide, and derivatives thereof.

Thus, the invention described herein makes possible the advantages of (1) providing an ophthalmic composition comprising a sleep adjusting substance which normalizes irregularity of the rhythm of sleep and a liquid ophthalmic medicament comprising the composition, the composition and the medicament being safe and obtainable by anyone, and (2) providing a method for regulating the rhythm of sleep comprising instilling the ophthalmic medicament including the sleep adjusting substance to an individual.

60 These and other advantages of the present invention will become apparent to those skilled in the art upon reading and understanding the following detailed description with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing a change with time in the locomotor activity of a mouse which is instilled with an

ophthalmic medicament prepared from an ophthalmic composition of the present invention including uridine.

FIG. 2 is a graph showing a change with time in the locomotor activity of a mouse which is instilled with an ophthalmic medicament prepared from an ophthalmic composition of the present invention including prostaglandin D<sub>2</sub> (PGD<sub>2</sub>).

FIG. 3 is a graph showing a change with time in the locomotor activity of a mouse which is dosed with an ophthalmic medicament prepared from an ophthalmic composition of the present invention including cytidine.

FIG. 4 is a graph showing a change with time in the locomotor activity of a mouse which is dosed with an ophthalmic medicament prepared from an ophthalmic composition of the present invention including delta-sleep-inducing peptide (DSIP).

FIG. 5 is a graph showing a change with time in the locomotor activity of a mouse which is dosed with an ophthalmic medicament prepared from an ophthalmic composition of the present invention including piperidine.

FIG. 6 is a graph showing a change with time in the locomotor activity of a mouse which is dosed with an ophthalmic medicament prepared from an ophthalmic composition of the present invention including oxidized glutathione (GSSG).

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Examples of the sleep inducing substances derived from an organism used in the present invention include uridine, sleep promoting substance (SPS), adenosine, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), delta-sleep-inducing peptide (DSIP), piperidine, 2-octyl-gamma-bromoacetate (gammabrom), arginine vasotocin (AVT), melatonin, serotonin, tryptophan and oxidized glutathione (GSSG).

Among the above-mentioned substances, SPS is a sleep inducing substance derived from a sleep deprivation mouse. The structure thereof is now being elucidated. Uridine is a part of an SPS molecule. It has been reported that an injection of only 10 pmole of uridine into a cerebral ventricle induces non-REM sleep (Honda et al., *Neurosci. Res.*, 1: 243 (1984)). Therefore, uridine is most appropriate in cases such as in the present invention where a small amount of the substance is expected to be transported into a brain by the instillation to the eye. Adenosine, which is relative to uridine, is also known to induce non-REM sleep by being administered into a lateral ventricle of a rat. PGD<sub>2</sub> is a strong sleep inducing substance discovered by Hayashi et al., and contrasts with PGF<sub>2</sub> which is also included in the prostaglandins but inhibits sleep (Osamu Hayashi, *J. Biol. Chem.*, 263: 14593 (1988)). Piperidine is one of the endogenous amines existing in the brains of mammals and is known to exhibit a sleep inducing activity (Miyata et al., *Life Sci.*, 15: 1135 (1974)). Moreover, piperidine is suggested to play an important role in hibernation. 2-Octyl-gamma-bromoacetate, which is commonly called gammabrom, is a REM sleep inducing substance extracted from human cerebrospinal fluid. Melatonin and AVT are pineal body hormones, the former is considered to adjust the biological rhythm, while the latter induces non-REM sleep in an animal and REM sleep in a human by increasing the amount of serotonin in the brain. Serotonin is a neurotransmitter that has been suggested to be concerned in sleep for a long time, and tryptophan is a precursor of serotonin. Oxidized glutathione (GSSG) was revealed by Komoda Y. et al. (*Chem. Pharm. Bul.*, 38: 2057 (1990)) as an essence of SPS-B, a part

of an SPS molecule as is uridine. Even a small amount of oxidized glutathione injected into a brain lengthens both non-REM sleep hours and REM sleep hours.

Examples of the sleep inhibiting substances derived from an organism used in the present invention include cytidine, prostaglandin E<sub>2</sub> and DSIP. Among these sleep inhibiting substances, cytidine has been reported to increase arousal hours and decrease total sleep hours when administered into a cerebral ventricle (Radulovacki et al., *Psychopharmacology*, 87: 136 (1985)). The sleep inhibiting activity of prostaglandin E<sub>2</sub> was found by Matsumura, a member of the Hayashi group. DSIP is a peptide comprising nine amino acids, and is known to induce sleep when administered into a cerebral ventricle of a rabbit (Schoenberger et al., *Proc. Natl. Acad. Sci. USA*, 74: 1282 (1977)). DSIP is also known to have an anti-stress activity such as causing sleep inhibition when administered to a human (Schnieder-Helmer et al., *Experientia*, 37: 913 (1981)).

Such sleep adjusting substances can be obtained by isolating and purifying the substance from an organism; by producing with a genetic engineering technique; or by chemical synthesizing.

The ophthalmic composition of the present invention can be obtained by combining the sleep adjusting substance with, if necessary, additives generally used in an ophthalmic medicament such as an isotonic agent, a buffer, and a preservative; and additives for enhancing the function of the used sleep adjusting substance. An example of the isotonic agent includes sodium chloride. Examples of the buffer include boric acid, sodium hydrogenphosphate and disodium hydrogenphosphate. Examples of the preservative include benzalkonium chloride, benzethonium chloride and chlorobutanol. Examples of the additives for enhancing the activity include a degrading enzyme inhibitor and a viscosity improver. The amount of each additive depends upon the kind of the used sleep adjusting substance and the additive. Moreover, the ophthalmic composition can contain the sleep adjusting substance alone or in combination with an additive except for the above.

The ophthalmic medicament comprising sleep adjusting substances derived from an organism is prepared by dissolving the ophthalmic composition of the present invention in sterilized purified water or saline; or dissolving the ophthalmic composition of the present invention in water or saline, followed by sterilization. The above-mentioned additives can be added at the time of dissolving the ophthalmic composition comprising the sleep adjusting substance alone in water or saline. The sleep adjusting substance derived from an organism is contained in such an ophthalmic medicament generally at a concentration of 1  $\mu$ M to 1 nM, preferably 10  $\mu$ M to 100  $\mu$ M.

When the ophthalmic medicament of the present invention is instilled, the sleep adjusting substance contained in the ophthalmic medicament is taken up by ganglion cells in the retina. The taken up substance is transported to a suprachiasmatic nucleus in the brain via a transporting system in the neuron called an axonal flow of a neural projection designated as a retina-hypothalamic tract. The suprachiasmatic nucleus is a part of the hypothalamus and is just above the optic chiasm. Especially in a mammal, the suprachiasmatic nucleus is regarded as a site where a biological clock exists. It is generally known that a functional site of a sleep promoting substance is somewhere between the hypothalamus and the lower part of the thalamus. Therefore, the suprachiasmatic nucleus, which is a part of the

hypothalamus, is considered to be concerned in the rhythm of sleep, which is an important element of one's circadian rhythm. Accordingly, the administered sleep adjusting substance works at this point, resulting in adjusting the sleep-wake rhythm through its center.

Moore et al. (*J. Comp. Neurol.*, 146: 1 (1972)) have reported on the retino-hypothalamic tract existing between the retina of an eyeball and the suprachiasmatic nucleus. According to a report by H. Nishino, when horseradish peroxidase, an enzyme with a molecular weight of about 40,000, which is frequently used as a tracer due to its histochemical dyes ability, injected into an eyeball, it is taken up by the ganglion cells, and then transported to the suprachiasmatic nucleus through the axonal flow (*Zoku Baforizuma to Sono Kiko (Japanese phonetics)*, Kodansha Scientific, 1978, pp. 203-214).

When the ophthalmic medicament comprising the ophthalmic composition of the present invention is administered, in order to exhibit its activity the sleep adjusting substance contained therein can be transported to the suprachiasmatic nucleus through other nerve conduction paths, or can directly affect neurons of the retina and the like. For example, it has been reported that uridine induces or inhibits excitation of the neurons, and can affect the nervous system distributed in the retina and the eyeball.

#### EXAMPLES

The present invention will now be illustrated by the following examples.

(Test method)

Three ICR male mice were put in a cage and bred under the condition of a day-and-night cycle of every 12 hours (the daytime from 7:35 to 19:35; the nighttime: from 19:35 to 7:35) for an experiment. Since the mice are nocturnal, they remain inactive until several tens minutes after the lights-out. When saline was administered to the mice by the instillation to the eye 30 minutes before the lights-out, the locomotor activity of the mice was temporarily increased. However, the activity faded within 30 minutes after the instillation and then disappeared. The group that had the saline instillation became active about 30 minutes later than the group without the saline instillation. Namely, the mice with the saline instillation became active about 20:10, which is about 1 hour after the administration of the saline.

In this manner, the mice were habituated to the instillation by administering the saline every day. After the same activity pattern appeared for two days continuously, an ophthalmic medicament containing a desired sleep adjusting substance was instilled for consecutive two days. The ophthalmic medicament was prepared by dissolving the sleep adjusting substance in saline. Thirty five minutes before the lights-out (at 19:00), about 5  $\mu$ l of the ophthalmic medicament comprising the sleep adjusting substance (100  $\mu$ M) was instilled into each eye of the mouse, respectively. The locomotor activity (unit: count) of mice was measured every 10 minutes by using a measuring device for the locomotor activity of an experimental animal "Automex" (produced by Tokai Irika). The locomotor activity was measured by putting a mouse on the measuring device. Every time any of the mice moved, a counter of the device cumulated the number of the movements. Thus, the larger cumulated number reflected more energetic activity. Such a measurement was conducted twice (for two days). An average value of the locomotor activity at each time was calculated and the results were plotted to obtain a graph, with time as the abscissas and the average locomotor activity as the ordinates. This graph depicts an activity pattern of the mice as

a function of an administration of the ophthalmic medicament. A total of the locomotor activity (i.e., an area below the curve in the graph) per one hour between 19:30 which is 30 minutes after the administration and 20:30 was calculated. In the same manner, as for the mice with the administration of saline alone, a total locomotor activity per the same one hour period as above was calculated. The ratio (%) of the calculated total locomotor activity per one hour of the mice with saline alone to that of the mice with the administration of the ophthalmic medicament (hereinafter called the "total activity ratio") was obtained. The mice whose activity patterns were not stable during the habituation to the administration of saline were not used in the following examples.

#### Example 1

##### [A sleep inducing activity by uridine]

Uridine was used as the sleep adjusting substance derived from an organism. The total activity ratio (%) is shown in Table 1, and the activity pattern of the mice with the instillation of the ophthalmic medicament containing 100  $\mu$ M of uridine is shown in FIG. 1. As is apparent from FIG. 1 and Table 1, the time when the mice started to move shifted backward in the group with the administration of uridine and the locomotor activity thereof was remarkably decreased.

Inoue et al. have reported that uracil, a component of uridine, does not have a sleep inducing activity (Honda et al., *Reports Med. Dent. Eng.*, 18: 93 (1984)). When an ophthalmic medicament including 100  $\mu$ M of uracil was instilled to a mouse, the locomotor activity of the mouse did not decrease (Table 1). Thus, the ophthalmic medicament including uridine showed a sleep inducing activity by the instillation.

Next, an ophthalmic medicament including 100  $\mu$ M of uridine was instilled to the ICR mice continuously for 2 weeks. There was no change in the weight gain of the mice as compared with the group without the instillation of uridine, and any change of an eyeball and/or internal organs was not visually observed.

TABLE 1

Change of the locomotor activity by uridine		
Compound	Concentration ( $\mu$ M)	Total Activity ratio per hour (% of control)
Uridine	10	63
Uridine	30	57
Uridine	100	53
Uracil	100	110

#### Example 2

##### [A sleep inducing activity by prostaglandin D<sub>2</sub> (PGD<sub>2</sub>)]

PGD<sub>2</sub> was used as the sleep adjusting substance. As is shown in FIG. 2, the time when the mice started to move shifted backward by the instillation of an ophthalmic medicament including 100  $\mu$ M of PGD<sub>2</sub>. The total activity ratio was reduced to 78% of the control group.

#### Example 3

##### [A sleep inhibiting activity by cytidine]

Cytidine was used as the sleep adjusting substance. The time when the mice started to move advanced by the instillation of an ophthalmic medicament including 100  $\mu$ M of cytidine, and the total activity rose to 127% (FIG. 3).

This suggests that the instillation of cytidine can inhibit sleep. This sleep inhibition is not caused by a pain or an itch in the eyes, supported by the fact that the locomotor activity completely disappeared temporarily after the instillation. Thus, there is a time lag between the installation of the sleep adjusting substance and the activity affected thereby, which is considered to be due to the time required to transport the substance through the axonal flow.

Example 4

[A sleep inhibiting activity by DSIP]

DSIP was used as the sleep adjusting substance. DSIP (100  $\mu$ M) was dissolved in saline containing 0.1% bovine serum albumin. The instillation of the resultant solution exerted a sleep inhibiting activity when measured in the same manner as in the above examples (FIG. 4), and the total activity ratio rose to 130%. This is regarded to show that an instillation, which is stressful for a mouse, was relieved by DSIP. The sleep inhibition is certainly not due to any pain or itch in the eyes for the same reason as was shown in Example 3 using cytidine.

Example 5

[A sleep inducing activity by piperidine]

Piperidine (100  $\mu$ M) was used as the sleep adjusting substance. The activity pattern is shown in FIG. 5, and the total activity ratio per hour was reduced to 72%.

Example 6

[A sleep inducing activity by GSSG]

GSSG (100  $\mu$ M) was used as the sleep adjusting substance. The activity pattern is shown in FIG. 5, and the total activity ratio per hour was reduced to 72%.

As described above, the ophthalmic composition comprising the sleep inducing substance or the sleep inhibiting substance both derived from an organism is provided by the present invention. It is possible to allow the sleep adjusting substance, which does not sufficiently exhibit its activity by the conventional oral administration and the like, to affect directly the center by the instillation of the ophthalmic medicament prepared from the ophthalmic composition

comprising the sleep adjusting substance. Thus, it is possible to administer a safe sleep adjusting substance in a simple and inexpensive manner.

Various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope and spirit of this invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description as set forth herein, but rather that the claims be broadly construed.

What is claimed is:

1. A method for regulating a rhythm of sleep comprising administering a sleep adjusting substance into eyes, wherein the sleep adjusting substance is at least one selected from the group consisting of uridine, cytidine, and derivatives thereof.

2. A method according to claim 1, wherein the sleep adjusting substance is at least one selected from the group consisting of uridine and derivatives thereof.

3. A method according to claim 1, wherein the sleep adjusting substance is at least one selected from the group consisting of cytidine and derivatives thereof.

4. A method for regulating a rhythm of sleep comprising administering an ophthalmic composition into eyes, wherein the ophthalmic composition comprises at least one of a sleep adjusting substance selected from the group consisting of uridine, cytidine, and derivatives thereof.

5. A method for regulating a rhythm of sleep comprising administering an ophthalmic medicament into eyes, wherein the ophthalmic medicament comprises an ophthalmic composition which contains at least one of a sleep adjusting substance selected from the group consisting of uridine, cytidine, and derivatives thereof.

6. A method according to claim 5, wherein the ophthalmic medicament is administered by instillation into eyes.

7. A method according to claim 5, wherein the sleep adjusting substance is contained in the ophthalmic medicament at a concentration of 1  $\mu$ M to 1 mM.

8. A method according to claim 5, wherein the sleep adjusting substance is contained in the ophthalmic medicament at a concentration of 10  $\mu$ M to 100  $\mu$ M.

\* \* \* \*

Related Proceedings Appendix

None